Remarks

Claims 1, 5, 15-37, 52-54, 77, 85, 88-90, 93, 98 and 107-120 were previously pending in this application.

Claims 1, 5, 15-37, 52-54, 77, 85, 88-90, 93, 98 and 107-120 are now cancelled without prejudice or disclaimer.

New claims 121-138 are added. Support for these new claims can be found at least in the originally filed claim set and in the specification as follows:

New Claim	Support
121	claims 47, 48, 50, 75; page 4 lines 28-30, page 12 line 20, page 14 lines 16-27, page 14 lines 28-31, page 15 lines 17, 16, 27, 28 and 31, page 16 lines 2, 5, 8 and 10, page 40 line 3 (SEQ ID NO:46), page 43 line 50 (SEQ ID NO:246), page 102 lines 1-6 and 21-22
122, 138	page 4 lines 28-30, page 12 line 20
123	page 28 lines 29-30, page 30 lines 5-6
124, 132	page 30 lines 5-7
125, 133	claim 18, page 6 lines 21-23
126, 134	claim 19, page 6 lines 21-23
127, 135	claim 21, page 6 line 23-24
128, 136	page 120 lines 9-10
129, 137	claim 52, page 65 lines 6-7
130	page 14 lines 19-22
131	page 15 line 31, page 16 line 8

Claims 121-138 are pending for examination with claim 121 being an independent claim. No new matter has been added.

Telephone Interview with Examiner Blanchard

Applicant's representative contacted the Examiner on June 2, 2006 to notify him of Applicant's intent to cancel the pending claims and introduce new claims of narrowed scope. Applicant's representative also indicated to the Examiner that the new claims are still within the group and species previously elected, as determined from a review of the restriction history in the case.

Rejection under 35 U.S.C. §112, first paragraph

Enablement

Claims 1, 5, 15-37, 52-54, 77, 85, 88-90, 93, 98, 107-112 and 118-120 are rejected under 35 U.S.C. §112, first paragraph, for lack of enablement. The rejected claims are now cancelled and the prior rejection should be moot. Applicant however presents a Wands analysis of the new claims in order to expedite prosecution.

The test of enablement is whether undue or unreasonable experimentation is required for one of ordinary skill in the art to practice (i.e., make and use) the claimed invention. Based on the specification and the knowledge in the art at the time of filing (i.e., effective filing date), one of ordinary skill must be able to make and use the claimed invention without undue experimentation. However the experimentation may be complex and still not be undue, if the art routinely engages in that level of experimentation. The factors to be considered in determining whether undue experimentation is required include 1) the nature of the invention; 2) the breadth of the claims; 3) the state of the art; 4) the level of ordinary skill in the art; 5) the level of predictability in the art; 6) the amount of direction provided by the inventor(s); 7) the existence of working examples; and 8) the quantity of experimentation needed to make or use the invention based on the content of the disclosure. In re Wands, 858 F.2d 731; 8 USPQ 2d 1400 (Fed. Cir. 1988). These factors are to be considered in their totality with no one factor being dispositive of the issue of enablement. Applicant analyzes each factor below in the context of the new claims.

Nature of the invention: The claimed invention relates to the use of T-rich oligonucleotides for the purpose of stimulating immune responses, including immune responses useful in the treatment of cancer. The T-rich oligonucleotides comprise a CpG motif. The oligonucleotides are used together with anti-cancer agents.

Breadth of the claims: The claimed invention is a method for treating a subject having cancer by administering an immunostimulatory oligonucleotide comprising the nucleotide sequence of SEQ ID NO:246 (independent of backbone composition) to a subject in combination with one or more particular anti-cancer agents.

Level of ordinary skill in the art: The level of skill in the art is that of a medical practitioner. The level of skill in the art inversely correlates with the amount of guidance and teaching that the Applicant must provide.

State of the art and predictability in the art: At the time of filing, the art was aware of immunostimulatory oligonucleotides, including their ability to induce cytokines and activate immune cells in vitro and to induce similar activities in vivo. The ability to make and use such oligonucleotides for immunostimulation was also known at that time.

Amount of direction provided by the inventor: The specification teaches the minimal 24 base consensus sequence shared by the claimed immunostimulatory oligonucleotides. The specification teaches how to formulate, dose and administer the immunostimulatory oligonucleotides. The recited anti-cancer agents were known and were commercially available. Their formulation, dosing and administration was also known in the art. MPEP § 2164.01(c) states that "if a statement of utility in the specification contains within it a connotation of how to use, and/or the art recognizes that standard modes of administration are known and contemplated, 35 U.S.C. 112 is satisfied." (citing In re Johnson 282 F.2d 370, 373 (CCPA 1960).) Accordingly, the amount of direction provided by the specification is considered sufficient, particularly in view of the level of ordinary skill in the art.

Working examples: The specification teaches, inter alia, the ability of an immunostimulatory oligonucleotide comprising the nucleotide sequence of SEQ ID NO:246 to induce high levels of NK lytic function (Example 2), to induce B cell proliferation, (Example 3), and to induce IL-6 and TNF-alpha secretion (Example 8). These activities, particularly NK lytic activity, have been associated with anti-tumor activity in vivo. Applicant provides post-filing references documenting results from human clinical trials that demonstrate anti-tumor activity of an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO:246 (i.e., CpG 7909, PF-3512676) when used in combination with carboplatin and paclitaxel in subjects having non-small cell lung cancer. These clinical experiments were carried out in accordance with the teaching provided in the specification. Subjects having non-small cell lung cancer were administered an

oligonucleotide of SEQ ID NO:246 by injection and in combination with carboplatin and paclitaxel.

Quantity of experimentation: The quantity of experimentation needed to make and use the invention, in view of the disclosure and the state of the art at the time of filing, is not beyond the level of experimentation routinely practiced by persons of ordinary skill in the art.

In view of the foregoing, Applicant submits that the newly added claims can be practiced without undue experimentation, and thus are enabled.

Written Description

Claims 113-117 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. Claims 113-117 are cancelled. Withdrawal of the rejection is respectfully requested.

Rejection under 35 U.S.C. §102(b)

Claim 120 is rejected under 35 U.S.C. §102(b) as anticipated by Jones et al. (Vaccine 17(23-24):3065-71, August 6, 1999). Claim 120 is cancelled. Withdrawal of the rejection is respectfully requested.

Jones et al. does not anticipate the newly added claims.

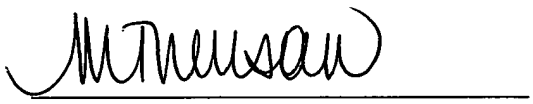
Objection under 37 CFR 1.75(c)

Claim 15 is objected to under 37 CFR 1.75(c) as being of improper dependent form, for failing to further limit the subject matter of a previous claim. Claim 16 is cancelled. Withdrawal of the rejection is respectfully requested.

Summary

In view of the foregoing amendments and remarks, this application should now be in condition for allowance. A notice to this effect is respectfully requested. If the Examiner believes, after this amendment, that the application is not in condition for allowance or has any questions or comments, he is requested to call the Applicant's representative at the telephone number listed below.

Respectfully submitted,



Maria A. Trevisan, Reg. No. 48,207
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2211
Telephone: (617) 646.8266

Docket No.: C1039.70035US00
Date: June 13, 2006
x06.13.06x

Therapeutic potential of Toll-like receptor 9 activation

Arthur M. Krieg

Abstract | In the decade since the discovery that mouse B cells respond to certain unmethylated CpG dinucleotides in bacterial DNA, a specific receptor for these 'CpG motifs' has been identified, Toll-like receptor 9 (TLR9), and a new approach to immunotherapy has moved into the clinic based on the use of synthetic oligodeoxynucleotides (ODN) as TLR9 agonists. This review highlights the current understanding of the mechanism of action of these CpG ODN, and provides an overview of the preclinical data and early human clinical trial results using these drugs to improve vaccines and treat cancer, infectious disease and allergy/asthma.

Pattern-recognition receptors
Receptors that bind to molecular patterns found in pathogens but not mammalian cells. Examples include the mannose receptor, which binds to terminally mannosylated and polymannosylated compounds, and Toll-like receptors, which are activated by various microbial products, such as bacterial lipopolysaccharides, hypomethylated DNA, flagellin and double-stranded RNA.

CpG motifs
DNA oligodeoxynucleotide sequences that include an unmethylated cytosine–guanosine sequence and certain flanking nucleotides, which have been found to induce innate immune responses through interaction with the Toll-like receptor 9.

Coley Pharmaceutical Group, Inc., 93 Worcester Street, Suite 101, Wellesley, Massachusetts 02481, USA.
e-mail: akrieg@coleypharma.com
doi:10.1038/nrd2059

Vertebrates are endowed with two complementary immune systems, the innate and the adaptive. The adaptive immune system is mediated by the highly sophisticated and recently evolved B and T cells, which specifically target the invader, and provide a memory response to prevent a repeat of the infection. The innate immune system, in contrast to the adaptive system, was relatively neglected for many decades until recent discoveries provided a remarkable new understanding of how it accomplishes its crucial mission. To protect the host from succumbing to infections, the innate immune system, which is evolutionarily more ancient than adaptive immunity, must accomplish four fundamental tasks (BOX 1). First, it must rapidly detect any infectious agent, regardless of whether it is a virus, bacteria, fungus or parasite. Second, innate immune cells seem to rapidly categorize the type of invading infectious agent as to whether it is located extracellularly or intracellularly. Third, innate immune defences appropriate to the pathogen class are activated to either eradicate or at least temporarily contain the infection. Fourth, innate immune cells induce the appropriate type of adaptive immune response to eliminate the infection and prevent its recurrence.

The key feature of innate immune cells that enables them to detect and categorize infection seems to be their repertoire of what have been termed pattern-recognition receptors (PRRs), which bind certain general types of molecules that are expressed across broad classes of pathogens, but which are absent or restricted in some way in vertebrates. The best understood family of PRRs are the Toll-like receptors (TLRs), of which 10 are known in humans (reviewed in REF. 1). TLRs that are specific for molecules characteristic of extracellular pathogens, such as lipopolysaccharides or lipopeptides, are expressed at the cell surface, whereas TLRs that detect intracellular pathogens are expressed within innate immune cells and are specific for nucleic acids. For example, TLR9 detects unmethylated CpG dinucleotides, which are relatively common in the genomes of most bacteria and DNA viruses, but which are suppressed and methylated in vertebrate genomes. The endosomal localization of TLR9 allows efficient detection of invading viral nucleic acids, while preventing 'accidental' stimulation by CpG motifs within self DNA[2]. Although beyond the scope of this review, it should be noted that a TLR9-independent cytosolic pathway of DNA detection has recently been demonstrated, perhaps indicating the importance of this capability for innate immunity[3,4].

Different immune cells express distinct subsets of the TLRs, which likely enables the immune system to tailor its responses against different pathogen classes[1]. Among resting human immune cells TLR9 is expressed primarily or exclusively in B cells and plasmacytoid dendritic cells (pDC), a specialized type of dendritic cell that produces most of the type I interferons (IFN) that are made in response to viral and intracellular pathogens[5] (reviewed in REF. 1). Some studies have also reported functional TLR9 expression in activated but not in resting human neutrophils[6] and pulmonary epithelial cells[7,8], but the biological significance of this TLR9 expression is uncertain.

Unfortunately for immunologists, the cellular patterns of TLR expression vary between different species. For example, mice differ from primates in that they express TLR9 not only in pDC and B cells, but also in monocytes and myeloid dendritic cells (reviewed in REF. 1).

Box 1 | **Roles of innate immunity**

Detection

Invading pathogens are detected by one or more members of several general families of 'pattern-recognition receptors' (PRRs), which include:

- TLRs (Toll-like receptors): 10 members known in humans, with diverse ligands
- NOD (nucleotide-binding oligomerization domain): detect muramyl dipeptide of peptidoglycan
- RIG1 (retinoic acid-inducible protein 1)-related proteins: detect dsRNA
- Mannose receptor: detects mannosylated lipoarabinomannans
- C-type lectins such as DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin): detect various antigens

PRRs detect molecules that have been called pathogen-associated molecular patterns (PAMPs); however, this term is slightly misleading, as none of these molecules are actually restricted in their expression to pathogens. Instead, what seems to distinguish a pathogen from a commensal organism is the anatomic or intracellular location of the molecule. For example, molecules from commensal flora would not be expected to stimulate a PRR on a basolateral epithelial surface, or to reach an intracellular PRR.

Categorization

Extracellular pathogens can be 'recognized' by cell-surface PRRs that bind broadly conserved structures such as flagellin (a highly conserved protein needed by motile organisms) and lipopolysaccharides. By contrast, detection of intracellular pathogens seems to be accomplished by intracellular receptors, including certain of the TLRs that are intra-endosomal, and RIG-I, which is cytoplasmic.

Containment

Depending on the type of infection, distinct subsets of innate immune cells produce cytokines and chemokines appropriate to limiting the spread of the infection. The response to an extracellular pathogen is typically dominated by pro-inflammatory cytokines such as tumour-necrosis factor-α (TNFα) and interleukin-12, whereas in the case of an intracellular pathogen the crucial innate immune products for control are the type I interferons.

Elimination and memory

The innate immune system can sometimes eradicate the infection on its own, in which case there will be no immune 'memory' of the event. Immune memory resides in adaptive immune cells, including both a humoral arm (B cells, which produce antibodies that can kill extracellular pathogens and prevent infection by intracellular pathogens) and a cellular arm (killer T cells, which are the most efficient killers of cells infected by intracellular pathogens).

This makes it difficult at best to predict accurately the effects of TLR9 activation in humans by extrapolating from results obtained in mice, in which more types of immune cells are activated by TLR9 agonists. This review will focus on the mechanisms and therapeutic applications of activating TLR9 with synthetic CpG oligodeoxynucleotide (ODN) agonists, which are currently in human clinical trials in the fields of infectious disease, cancer and asthma/allergy.

Targeted immune activation by CpG ODN

Most types of immune cells do not express TLR9, and so are not activated directly by CpG DNA. All of the cellular immune effects of CpG ODN in humans are thought to result directly and indirectly from activating TLR9-expressing pDC and B cells (BOX 2). pDC activated through TLR9 secrete IFNα, which drives the migration and clustering of pDC in the marginal zone and outer T-cell areas of the lymph node, where they are better able to stimulate adaptive immune responses[9]. TLR9-stimulated B cells and pDC show increased expression of co-stimulatory molecules, resistance to apoptosis, upregulation of the chemokine (C-C motif) receptor CCR7, and secretion of T_H1-promoting chemokines and cytokines such as monocyte inflammatory protein-1 (MIP1), IFNγ-inducible 10-kDa protein (IP10) and other IFN-inducible genes[10]. Co-activation of naive, germinal centre or memory B cells through the B-cell-antigen receptor and TLR9 induces their differentiation into plasma cells[11]; for memory B cells, activation through TLR9 alone is sufficient to drive differentiation to plasma cells[12,13]. The B-cell-stimulatory effect of TLR9 is so great that the efficiency of hybridoma generation from purified primary human memory B cells is improved from 1–2% without CpG to 30–100% with the addition of a CpG ODN[14]. Although CpG-induced plasma cell differentiation does not require T-cell help, its efficiency is enhanced by interactions with pDC, together with B-cell receptor crosslinking[15]. The net effect of TLR9 activation is to induce T_H1-biased cellular and humoral effector functions of innate and adaptive immunity (TABLE 1).

Even before the discovery that TLR9 was an intracellular protein, it was apparent that stimulation of immune cells by CpG ODN requires internalization[16]. ODN internalization occurs spontaneously in culture without the need for uptake enhancers or transfection, is temperature- and energy-dependent, and seems to be relatively sequence-independent. The earliest steps in the CpG-induced signal transduction pathways can be blocked by inhibitors of endosomal acidification/maturation[17–20] or by inhibitors of phosphatidylinositol 3-kinase, which seems to have a role in ODN internalization[21]. Following internalization into an endosomal compartment, CpG motifs seem to be bound and recognized by TLR9, leading to the rapid recruitment and/or activation of the adaptor molecules MyD88, interleukin-1 receptor-associated kinase-1 (IRAK1), interferon regulatory factor-7 (IRF7), and tumour-necrosis factor-α receptor activated factor-6 (TRAF6)[18,22–26]. This results in the rapid activation of several mitogen-activated protein kinases, including extracellular receptor kinase (ERK), p38, and Jun N-terminal kinase, as well as the IκB complex, and these pathways converge on the nucleus to alter gene transcription[27–33].

Structure–activity relationships of CpG ODN

The two bases to the 5′ and 3′ sides of the CpG dinucleotide comprise a CpG motif, one of which is sufficient for immune stimulation[16]. Early empirical structure–activity relationship (SAR) studies revealed species-specific differences in the optimal CpG motif, which is GACGTT for mice[16,34,35] but GTCGTT for humans[27]. Once TLR9 was identified as the CpG receptor, it was possible to show that the species-specific recognition was present at the level of the TLR9 itself[36] and that TLR9 directly binds DNA[26,37].

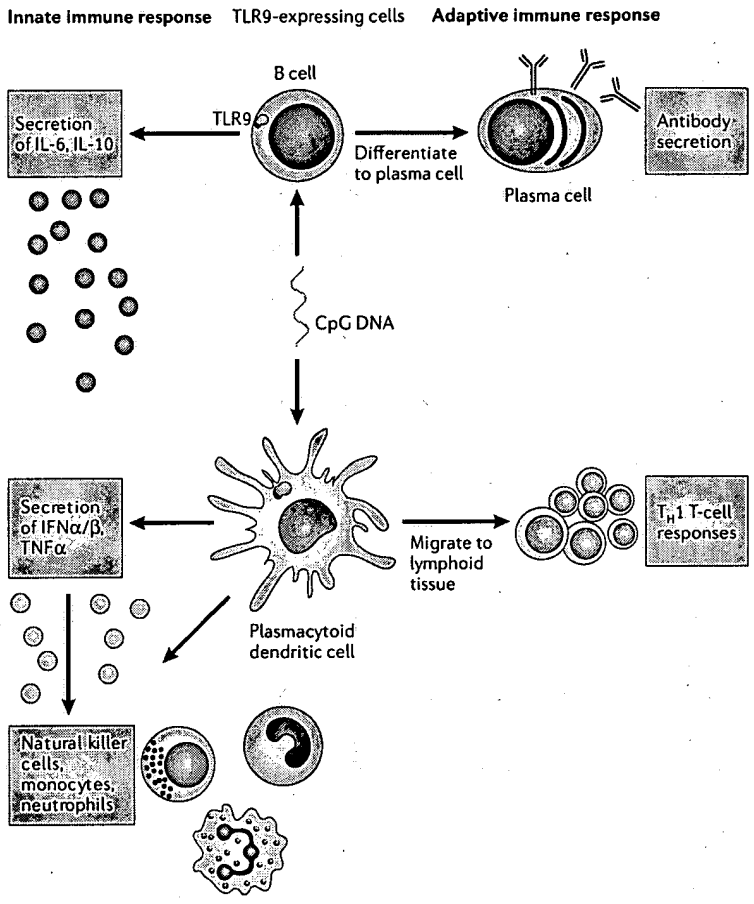
Besides the hexamer CpG motif, the immune-stimulatory activity of an ODN is determined by the number of CpG motifs it contains (usually two to four are optimal), the spacing of the CpG motifs (usually at least two intervening bases, preferably thymine residues, is optimal), the presence of poly-G sequences or other flanking sequences in the ODN (effect depends on

Plasmacytoid dendritic cell (pDC). A unique type of dendritic cell. These cells are also known as interferon (IFN)-producing cells because they are the main source of type I IFNs (such as, IFNα and IFNβ) during viral infections.

Co-stimulatory molecules Soluble or membrane-bound molecules that signal to T cells (or other immune cells) and, having little or no effect alone, either enhance or modify the physiological effect of the primary signal, which is mediated by engagement of the T-cell receptor (or other receptors on other immune cells).

Box 2 | **Role of TLR9 in triggering innate and adaptive immunity**

Two types of human immune cells express Toll-like receptor 9 (TLR9): B cells and plasmacytoid dendritic cells (pDC, see figure). Regardless of the presence or absence of CpG motifs, DNA is endocytosed into a cellular compartment where it is exposed to TLR9. If the DNA contains CpG motifs, then TLR9 is activated. In pDC, TLR9 activation is dependent on interleukin (IL)-1 receptor-associated kinase (IRAK)-4 and interferon regulatory factor-7 (IRF7), and requires direct interactions between IRF7 and MyD88, tumour-necrosis factor-α (TNF-α) receptor activated factor-6 (TRAF6) and IRAK-1[186–191]. TLR9 activation induces nuclear factor-κB (NF-κB) and other intracellular signalling pathways that initiate a rapid innate immune response that is characterized by the secretion of a variety of proinflammatory and antiviral cytokines, such as IL-6, TNFα and type I interferons (IFN), and immune regulatory cytokines that limit the intensity of the inflammatory response, such as IL-10. There is also a reverse effect of CpG-activated B cells on dendritic cells, by which TLR9 activation drives $CD5^+$ B cells to produce IL-10, which then suppresses the T_H1-priming function of the dendritic cells[192]. In contrast to some other innate immune responses, relatively little IL-12 is produced by TLR9-activated human immune cells. Natural killer (NK) cells and other innate immune cells are activated secondarily by pDC through both IFN-dependent and IFN-independent pathways. B cells activated through TLR9 have a greatly increased sensitivity to antigen stimulation, promoting their differentiation to antibody-secreting plasma cells, and therefore contributing to the adaptive immune response. TLR9-stimulated pDC migrate to the T-cell zones of lymph nodes and other secondary lymphoid tissues, express increased levels of co-stimulatory molecules that enhance their capacity to activate naive and memory T cells, and have increased capacity to cross-present soluble protein antigens to CD8 T cells. As a consequence, CpG DNA promotes strong T_H1 CD4 and CD8 T-cell responses.



ODN structure and backbone), and the ODN backbone (a nuclease-resistant phosphorothioate backbone is the most stable and best for activating B cells, but gives relatively weaker induction of IFNα secretion from pDC compared with native phosphodiester linkages in the CpG dinucleotide)[16,27,35,38–41]. In addition, the immune-stimulatory effects of the ODN are enhanced if there is a TpC dinucleotide on the 5′ end and if the ODN is pyrimidine rich on the 3′ side[27,35,39,42].

For therapeutic applications CpG ODN are typically synthesized with at least partially phosphorothioate-modified (PS-ODN) backbones to provide nuclease resistance and increased half-life, and generally produce a greater immune-stimulatory effect. There are at least three classes of immune-stimulatory CpG ODN with distinct structural and biological characteristics (BOX 3). The capacity of the different CpG ODN classes to induce such divergent immune effects might seem surprising, because these effects all seem to result from the activation of a single receptor, TLR9[43,44]. Maximal induction of pDC IFNα secretion is associated with ODN that can form secondary structures, such as the dimeric C class and the multimeric A class. These higher-ordered structures might induce TLR9 crosslinking, promote the recruitment of one or more additional cofactors or adaptor proteins into the TLR9 signalling complex, and/or alter the intracellular compartmentalization of the ODN[45]. It therefore seems that the biological activity of TLR9 can be modulated by designing ligands that bind it in different ways.

A wide range of DNA backbones, modifications and substitutions have been explored for their effects on the capacity of ODN to activate TLR9 and induce innate and adaptive immunity. These SAR studies have shown that such modifications can alter the magnitude and character of immune activation, within the range of effects shown for the different ODN classes described above (reviewed in REFS 46,47). TLR9 recognition of the CpG motif in a phosphorothioate backbone seems to be sensitive to the P chirality, with the R stereoisomer preferred[48].

Several types of suppressive ODN (S-class ODN) have been shown to block TLR9 signalling, but do not block activation of immune cells through TLR4, CD40 or the B-cell receptor[49–51]. In contrast to original expectations, S-class ODN are not specific TLR9 inhibitors; some also block TLR7 and/or TLR8 and RNA-mediated immune activation, depending on the presence or absence of specific sequence motifs[52–54]. Although incompletely understood, the mechanism of suppression by S-class ODN is distal to ODN uptake, proximal to early signalling

Plasma cells
Non-dividing, terminally differentiated immunoglobulin-secreting cells of the B-cell lineage.

Table 1 | **Activation of both innate and adaptive humoral and cellular immunity by TLR9 agonists**

Immune system	Humoral effects	Cellular effects
Innate	• IFNα secretion • Secretion of IFN-inducible chemokines and cytokines • Secretion of pro-inflammatory cytokines (IL-6, TNFα) • Secretion of anti-inflammatory cytokines (IL-10, IL-1RA)	• NK cell lytic activity • Monocyte expression of TNF-related apoptosis-inducing ligand (TRAIL)[198] • Neutrophil activation, migration and bacterial uptake[70,199,200]
Adaptive	• Induction of IgG isotype switching and antibody secretion • Suppression of IgE antibody production	• Differentiation of T_H1 cells • Enhanced cross-priming • Increased CTL

CTL, cytotoxic T lymphocyte; IFN, interferon; IL-10, interleukin-10; Ig, immunoglobulin; IL-1RA, interleukin-1 receptor antagonist; NK, natural killer; TLR9, Toll-like receptor 9; TNFα, tumour-necrosis factor-α.

events such as nuclear factor-κB (NF-κB) activation, and could involve direct blockade of the signal transduction cascade leading to interleukin-12 (IL-12) and IFNγ production[51,55]. SAR studies have revealed that to most effectively block CpG-induced TLR9 activation, S-class ODN should contain a CCT motif near the 5′ end, and at least one G-rich region three to five bases 3′ to this[56].

Drug-like properties of synthetic CpG ODN

Some of the characteristics of synthetic ODN are quite attractive for drug development, whereas others are less favourable (BOX 4). The technology for commercial-scale (multi-kilogram) ODN synthesis and purification, carried out according to Good Manufacturing Practices, has been well developed during the past decade of antisense and aptamer drug development. Antisense and aptamer oligonucleotide drugs have been approved by the US FDA, establishing a regulatory pathway for this general class of drugs. The absorption, distribution, metabolism and elimination (ADME) properties of synthetic PS-ODN with and without CpG motifs have been well characterized and reported in the extensive literature on antisense ODN, which has shown these characteristics to be essentially sequence-independent[57,58]. ODNs given subcutaneously are slowly absorbed from injection sites (with the highest concentration in the draining lymph nodes for the first several days after injection), and then enter the systemic circulation, where they demonstrate high-capacity, low-affinity binding to plasma proteins, principally albumin. ODN are rapidly cleared into tissues, especially the liver, kidneys and spleen, but do not seem to cross the blood–brain or blood–testes barriers. Catabolism of ODN typically occurs by exonuclease digestion and base clipping, primarily at the 3′ end, resulting in natural DNA bases and thiophosphate metabolites that are excreted in the urine. The immune effects of CpG ODN administration through different routes result from their ADME characteristics. For example, subcutaneous administration of CPG 7909 (Coley), which results in high levels of the compound in the draining lymph node (which would contain a relatively high concentration of TLR9-expressing cells), induces high levels of serum cytokines and chemokines[59]. On the other hand, even relatively high-dose intravenous administration of CPG 7909, which is rapidly diluted in the blood and is approximately 95% protein bound, fails to induce measurable serum cytokine responses in humans[59]. Because the pharmacodynamics of subcutaneous CpG ODN result from the local ODN concentration in the draining lymph nodes, they do not match the systemic pharmacokinetics.

Therapeutic applications of CpG ODN

Prevention and therapy of infectious disease. If the normal function of TLR9 is to stimulate protective immunity against intracellular pathogens, then it could be proposed that prophylactic or therapeutic treatment with a synthetic TLR9 ligand would provide protection against an intracellular infectious challenge and/or eliminate a chronic infection. Indeed, studies in mice have demonstrated that the innate immune defences activated by CpG ODN given by injection, inhalation or even by oral administration can protect against a wide range of viral, bacterial and even some parasitic pathogens, including lethal challenge with Category A agents or surrogates such as *Bacillus anthracis*, vaccinia virus, *Francisella tularensis* and Ebola virus[60–77]. The mechanisms of protection have only been partially investigated. Protection in a *Listeria monocytogenes* model has been linked to CpG-activated dendritic cells, which protect naive mice against adoptive transfer[78–80]. Additional cell types might also provide some protection, because naive mice that received CpG-pretreated spleen cells depleted of $CD11c^+$ dendritic cells still had a partial survival benefit. The parameters of protection in the different models vary widely — protection lasts for at least 2 weeks after a single CpG dose in models such as *L. monocytogenes* and *F. tularensis* (LVS strain)[60,63,64], but only for a day or so in the vaginal herpes simplex virus challenge model[81].

Post-exposure therapy with TLR9 activation is generally ineffective against rapidly progressive acute infectious agents. However, TLR9 activation rapidly suppresses hepatitis B virus (HBV) replication in transgenic mice[82], suggesting potential utility of this approach in the treatment of chronic viral infections in humans. The antiviral effect in this model seemed to be indirect and secondary to CpG-induced IFNα secretion, because hepatocytes do not express TLR9 and viral replication was not suppressed in mice genetically deficient in the type I IFN receptor. TLR9 activation leads to improved survival when given four days post-infection in a Friend leukaemia virus model[76], and even when given more than 1 week after an indolent *Leishmania major* challenge[62]. Protective effects of CpG against *Leishmania* infectious challenge are not limited to rodents, but have also been observed in rhesus macaques, which were protected

Adoptive transfer
An experimental method in which lymphocytes from an antigen-primed donor mouse are introduced into an unprimed recipient mouse.

Box 3 | **Distinct classes of CpG oligodeoxynucleotides**

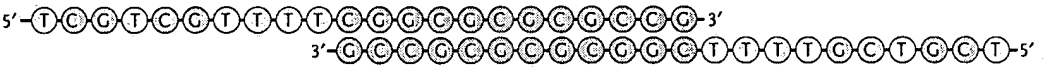
A-class ODN 2216

- CpG are phosphodiester, in palindrome
- Strongly induce pDC IFNα secretion
- Moderately induce pDC maturation
- Poorly induce B-cell proliferation

B-class ODN PF-3512676

- Phosphorothioate backbone, linear
- Strongly induce B-cell proliferation and pDC maturation
- Poorly induce pDC IFNα secretion

C-class ODN 2395



- Phosphorothioate backbone, 3′ palindrome forms duplex
- Has combined intermediate effects of both the A- and B-classes

Three major classes of CpG ODN that are structurally and phenotypically distinct have been described. Examples of each class are shown in the figure, using the ID numbers from the published reports (PF-3512676 formerly was also known as ODN 2006 and CPG 7909), together with the immune effects and structural characteristics that are specific to the class. The A-class CpG ODN (also referred to as type D) are potent inducers of interferon-α (IFNα) secretion (from plasmacytoid dendritic cells), but only weakly stimulate B cells. The structures of A-class ODN include poly-G motifs (three or more consecutive guanines) at the 5′ and/or 3′ ends that are capable of forming very stable but complex higher-ordered structures known as G-tetrads, and a central phosphodiester region containing one or more CpG motifs in a self-complementary palindrome. These motifs cause A-class ODN to self-assemble into nanoparticles[193]. B-class ODN (also referred to as type K) have a completely phosphorothioate backbone, do not typically form higher-ordered structures, and are strong B-cell stimulators but weaker inducers of IFNα secretion[10]. However, if B-class CpG ODN are artificially forced into higher-ordered structures on beads or microparticles, in dendrimers or with cationic lipid transfection, they exert the same immune profile as the A-class CpG ODN, thereby linking the formation of higher-ordered structures to biological activity[45,193–195]. The C-class CpG ODN have immune properties intermediate between the A and B classes, inducing both B-cell activation and IFNα secretion[43,196,197]. These properties seem to result from the unique structure of these ODN, with one or more 5′ CpG motifs, and a 3′ palindrome, which is thought to allow duplex formation within the endosomal environment.

against *Leishmania amazonensis* infection by treatment with 0.5 mg of A-class (D type) ODN 3 days before and 3 days after challenge, but were not protected by B-class (K type) ODN[83]. This protection was even seen in macaques infected with simian immunodeficiency virus, which showed similar levels of immune response to CpG stimulation to normal animals.

Hepatitis C virus (HCV) infection is cleared spontaneously in about 20% of people who become infected, but becomes chronic in the remainder, which includes approximately 170 million people worldwide. There is no highly effective therapy for chronic HCV infection; fewer than half of North American patients respond to the current standard of care treatment, which is 48 weeks of a combination therapy with IFNα and ribavirin, which has a relatively high frequency of intolerable side effects. Spontaneous viral clearance has been associated with early and strong innate immune activation, which generates a strong and diverse T_H1 and CD8 cytolytic T-cell response against the virus, demonstrating the potential for an immune response to control the virus[84]. TLR9 activation can drive a similar pattern of innate and adaptive immune responses, and so a C-class CpG ODN, CPG 10101 (Coley), was investigated for its activity as a monotherapy against HCV. In a 4-week Phase Ib blinded randomized controlled trial involving 60 HCV-infected subjects, CPG 10101 caused a dose-dependent decrease in blood viral RNA levels[85]. At the highest dose level of 0.75 mg per kg weekly, there was up to a 1.6 mean maximum log reduction in viral RNA, which was associated with biomarkers for TLR9 activation, including natural killer cell activation and serum IFNα and IFN-inducible chemokines. Treatment was generally well tolerated, with the most common side effects being mild to moderate flu-like symptoms and reactions at the injection site, and the maximal tolerated dose was not reached. Further studies involving more sustained dosing and combination therapy with pegylated IFNα and ribavirin are underway to determine whether the immune activity of CPG 10101 can produce greater patient response rates than achieved with current therapies.

There are few reported experimental models in which pretreatment with CpG exacerbates infection. However, the immune expansion induced by TLR9 activation in rodents increases the number of susceptible target cells for Friend leukaemia virus, resulting in a more aggressive infection following challenge several days later[86], and CpG priming shortened survival slightly in a *Candida albicans* challenge model, in which T_H1 cytokines are detrimental[87]. In addition, TLR9 activation with bacterial

Box 4 | **Characteristics of CpG oligodeoxynucleotides**

Drug-like characteristics

- Excellent aqueous solubility
- Spontaneous intracellular uptake by certain immune cells (including especially those that express Toll-like receptor 9 (TLR9))
- Relatively simple solid-phase Good Manufacturing Practice synthesis (multi-kilogram scale) and chromatographic purification
- Comparatively well-understood chemistry enables diverse studies of structure–activity relationships
- Metabolites are mostly normal components of DNA, not novel small molecules
- Range of backbones available for modulating compound stability for different applications
- Can be administered through virtually any drug route (including oral)
- Dose exposure required for immune stimulation is ~0.1–1% of that required for antisense applications
- Excellent stability in aqueous solutions at physiologic pH, even at room temperature
- Well-developed highly analytic methods for Chemistry, Manufacturing and Controls (liquid chromatography–mass spectrometry is state of the art)
- Very sensitive methods available for detection of 'cold' compound[201]

Non-drug-like characteristics

- Medium size: molecular mass ~6,000-8,000 Da (length typically 18-25 bases)
- Highly charged polyanions
- Phosphorothioate and some other backbones are chiral
- Poor stability of purines in acid solution
- Cleaved by nucleases in serum or cell extracts (phosphorothioate backbone is relatively nuclease resistant)
- Highly protein bound
- Non-uniform organ distribution; highest tissue levels in kidney, liver and spleen after systemic delivery
- Pharmacokinetics do not match pharmacodynamics after subcutaneous delivery
- Sequence-independent effects, including concentration-dependent activation of complement proteins and prolongation of partial thrombin time

DNA can induce the HIV transcriptional regulatory elements in long terminal repeats (LTRs)[88], increasing viral replication. HIV-infected humans treated with a B-class ODN that contained a CpG motif showed dose-dependent increases in plasma HIV branched DNA levels, which represent the level of virus in the blood[89]. Because of the possibility of inducing increased HIV expression, CpG ODN therapy of HIV-infected individuals should probably only be undertaken during HAART (highly active antiretroviral treatment), unless the therapy is part of a clinical trial strategy to induce anti-HIV immunity. Despite their capacity to induce HIV transcription, CpG ODN can also show anti-HIV activity: the high level of IFNα production induced by A-class ODN suppresses HIV replication in human foetal thymus cells[90], and B-class ODN can also suppress HIV replication in cultured human cells, albeit in a sequence-independent fashion[91]. HIV-infected long-term non-progressors have much stronger natural killer cell activation in response to A-class CpG ODN compared with progressors[92], but it is not clear whether this difference in CpG-responsiveness is a cause or a consequence of the patient's clinical status. As will be discussed further below, a B-class CpG ODN has been used as a vaccine adjuvant in HIV-infected humans on HAART with no apparent increase in HIV expression[93], providing support for the cautious application of TLR9-based immunotherapeutic approaches.

Adjuvant
An agent mixed with an antigen that enhances the immune response to that antigen upon immunization.

Enhancing vaccines with TLR9 agonists. TLR9 activation enhances antigen-specific humoral and cellular responses to a wide variety of antigens, including peptide or protein antigens, live or killed viruses, dendritic cell vaccines, autologous cellular vaccines and polysaccharide conjugates in both prophylactic and therapeutic vaccines in numerous animal models. The mechanisms that contribute to the strong adjuvant activity of CpG ODN potentially include synergy between TLR9 and the B-cell receptor, which preferentially stimulates antigen-specific B cells[16]; inhibition of B-cell apoptosis[35]; enhanced immunoglobulin G (IgG) class switch DNA recombination[94–96]; and dendritic cell maturation and differentiation, resulting in enhanced activation of T_H1 cells and strong cytotoxic T-lymphocyte (CTL) generation, even in the absence of CD4 T-cell help[97,98]. Conjugation of a CpG ODN directly to an antigen can enhance antigen uptake and reduce antigen requirements[99,100], but cysteine residues in peptides or proteins can also form spontaneous disulphide bonds with the phosphorothioate linkage in ODN, resulting in enhanced CTL responses without the difficulties of a separate conjugation step[101].

Comparisons of different adjuvants in mouse models have demonstrated CpG ODN to be unsurpassed at inducing T_H1-type responses[102–105]. The T_H1 bias induced by TLR9 stimulation is maintained even in the presence of vaccine adjuvants such as alum or incomplete Freund's adjuvant (IFA) that normally promote a T_H2 bias[94,106,107]. Likewise, CpG ODN can overcome the T_H2 bias associated with a respiratory syncytial virus vaccine[108], and with vaccination in both very young and elderly mice[109–116]. CpG ODN show even greater adjuvant activity when formulated or co-administered with other adjuvants or in formulations such as microparticles, nanoparticles, lipid emulsions or similar formulations, which are especially necessary for inducing a strong response when the antigen is relatively weak[117]. CpG ODN are also effective mucosal vaccine adjuvants for respiratory tract[118–121], vaginal mucosal[122], oral or intrarectal vaccination[121,123–125], conjunctival vaccination[126] and even for transcutaneous immunization[127]. Vaccination through mucosal routes has succeeded in inducing both local and systemic humoral and cellular immune responses, including enhanced protection against infectious challenge[119,128].

In humans, CpG ODN have been used as adjuvants for hepatitis B vaccination either in combination with alum[129] or alone[130]. In a randomized, double-blind controlled Phase I/II dose-escalation study, healthy individuals received three intramuscular injections (using the FDA-approved vaccination regimen of 0, 4 and 24 weeks) of an alum-absorbed HBV vaccine either in saline or mixed with a B-class ODN, CPG 7909, at doses of 0.125, 0.5 or 1.0 mg[129]. Hepatitis B surface antigen (HBsAg)-specific antibody responses (anti-HBs) appeared earlier and had higher titres at all time points from 2 weeks after the initial prime up to 48 weeks in CPG 7909 recipients compared with those individuals who received vaccine

alone. Moreover, most of the subjects who received CPG 7909 as adjuvant developed protective levels of anti-HBs IgG within just 2 weeks of the priming vaccine dose, compared with none of the subjects receiving the commercial vaccine alone[129]. In this study, the addition of the TLR9 agonist also increased the proportion of antigen-specific high-avidity antibodies, suggesting enhancement of the late-affinity maturation process in the activated B cells[131].

The capacity of CPG 7909 to accelerate seroconversion was also demonstrated when it was used as an adjuvant to the approved anthrax vaccine in a randomized controlled trial in healthy volunteers. Control subjects reached their peak titre of toxin-neutralizing antibody at day 46, but this titre was already achieved in the subjects receiving CPG 7909 at day 22, more than 3 weeks earlier[132]. More rapid seroconversion to the anthrax toxin could be of great importance in the setting of a bioterrorist attack. Furthermore, the addition of CPG 7909 induced a statistically significant 8.8-fold increase in the peak titre of toxin-neutralizing antibody, and increased the proportion of subjects who achieved a strong IgG response to the anthrax protective antigen from 61% to 100%[132]. These results indicate great potential for TLR9 agonists as vaccine adjuvants in both mice and humans, despite the differences in immune cells expressing TLR9 between these species.

Certain populations are hyporesponsive to vaccination, especially immune-suppressed individuals such as those infected with HIV. A randomized double-blind controlled trial in HIV-infected humans who previously had failed to respond to an HBV vaccine, Engerix-B, alone demonstrated that addition of CPG 7909 to the vaccine significantly enhanced both the mean titres of anti-HBs and the antigen-specific T-cell proliferative response[93]. Perhaps of equal import, the proportion of HIV patients who had seroprotective levels at 12 months following vaccination was increased from 63% in the controls to 100% in the group receiving CPG 7909[93]. Moreover, with CPG 7909 the protective antibody levels and the significantly enhanced antigen-specific lymphocyte proliferation were maintained for more than a year[93].

The use of CpG ODN as a vaccine adjuvant in mice enables the antigen doses to be reduced by approximately two orders of magnitude, with comparable antibody responses to the full-dose vaccine without CpG[133]. In a Phase Ib randomized, double-blind controlled clinical trial, subjects vaccinated with a one-tenth dose of a commercial trivalent killed split influenza vaccine (Fluarix; GlaxoSmithKline) had reduced levels of antigen-specific IFNγ secretion from re-stimulated peripheral blood mononuclear cells (PBMC) compared with those measured in PBMC from subjects vaccinated with the full-dose vaccine alone[134]. However, the co-administration of CPG 7909 with the one-tenth dose of Fluarix restored the antigen-specific IFNγ secretion to the level seen with full-dose vaccine[134].

The T_H1-biased immune effect of CpG ODN has been applied to the development of allergy vaccines, which in mice are able to redirect the allergic T_H2 response and prevent inflammatory disease manifestations, even in mice with established allergic disease[135,136]. A conjugate of a CpG ODN to a portion of the ragweed allergen has been evaluated as an allergy vaccine in human clinical trials, which provided encouraging evidence for a selective and specific redirection of the allergic T_H2 response towards a non-allergic and non-inflammatory T_H1 response, and a significant clinical benefit with reduced allergic symptoms[137,138].

In a small Phase I tumour vaccine trial using a 1-mg dose of CPG 7909 as adjuvant to recombinant melanoma antigen family A, 3 (MAGEA3) tumour antigen for tri-weekly vaccination in six patients with metastatic melanoma, there were two stable disease and two partial responses beginning after seven to ten vaccinations, and lasting at least a year as assessed by by RECIST (Response Evaluation Criteria In Solid Tumors)[139]. In eight melanoma patients, CPG 7909 at a dose of 0.5 mg stimulated strong and rapid CD8 T-cell responses to a Melan-A tumour peptide antigen when used with Montanide (Seppic) as a cancer vaccine adjuvant[140]. Taken together, the results from these human clinical trials show that stimulation of TLR9-expressing cells (presumably pDC and B cells) is sufficient to induce strong and sustained humoral and cellular memory immune responses, even in those with HIV infection, allergy or cancer, offering several advantages over conventional vaccines (TABLE 2, BOX 5).

Directing adaptive immunity without a vaccine. Historically, induction of effective antigen-specific immune responses has required a vaccine. However, there are several therapeutic fields in which TLR9 activation has been applied to achieve a similar effect, but without a vaccine. For example, although allergy vaccines with CpG ODN typically provide rapid redirection of allergic responses, inhaled CpG ODN monotherapy given repeatedly can prevent or treat allergic airway responses not only in mouse models[141] but also in primates[142]. Potential mechanisms that have been proposed to explain the somewhat counterintuitive anti-inflammatory effect of TLR9 stimulation on pulmonary inflammation include the induction of a T_H1-like cytokine milieu that suppresses the T_H2 response, systemic expression of IL-10 or transforming growth factor-β (TGFβ), and pulmonary expression of indoleamine (2,3)-dioxygenase (IDO)[143,144] (TABLES 1,2).

CpG ODN have antitumour activity in many mouse models (reviewed in REF. 145). In relatively small tumours CpG monotherapy can be sufficient to induce a T-cell-mediated rejection of established tumours; however, to induce rejection of larger tumours the CpG ODN often needs to be combined with other effective antitumour strategies, such as monoclonal antibodies, radiation therapy, surgery and chemotherapy. Encouraging evidence for the capacity of TLR9 activation to induce a T_H1-like cytokine response in human cancer patients has been reported recently in studies of dendritic cells isolated from primary human tumours[100] and in lymphoma patients treated with a CpG ODN alone or together with an antitumour antibody[146,147]. Chemotherapy has historically been considered to be immune suppressive, so it

Seroconversion
Development of a detectable concentration of pathogen-specific antibodies in the serum as a result of infection or immunization.

Table 2 | **Therapeutic applications for TLR9 agonists**

Therapeutic approach	Animal models	Human clinical trials	Proposed mechanism of action
Infectious disease			
Monotherapy	Many, especially against viruses and intracellular bacteria, reviewed in REF. 200	• C-class ODN CPG 10101[85] in Phase II (Coley) for hepatitis B	Innate immune activation, with T_H1-like cellular and cytokine/chemokine responses
Vaccines	Many, reviewed in REF. 202	• B-class ODN 1018 ISS (Phase III; Dynavax) and CPG 7909 (Phase I; GlaxoSmithKline (GSK)/Coley and DARPA/NIAID/Coley) for hepatitis B[93,129,130,203], influenza[134], anthrax[132] and other indications	Enhancing antigen-specific humoral and cellular adaptive immune responses
Cancer			
Monotherapy	Many (especially intratumoral injection) reviewed in REF. 145	• B-class ODN PF-3512676[146] (Phase I; Pfizer/Coley)	NK-cell mediated in B16 IP melanoma model, T-cell mediated in most other models
Vaccines	Many, including peptide or protein antigens, carbohydrate conjugates, whole cell vaccines and DC vaccines, reviewed in REF. 145	• B-class ODN PF-3512676 (Phase I; Pfizer/Coley and GSK/Coley) with Melan-A peptide[140]; and with MAGE recombinant protein[139]	CD4 and/or CD8 T-cell mediated
Combination therapies	Various, including chemotherapy, radiotherapy, surgery, immunotherapy; reviewed in REF. 145	• B-class ODN 1018 ISS + Rituximab for NHL[147] (Phase I; Dynavax) • PF-3512676 combined with taxane/platin chemotherapy for NSCLC[153] (Phase III; Pfizer/Coley) • HYB-2055 in combination with gemcitabine and carboplatin for refractory solid tumours (Phase II; Idera)	TLR9 stimulation enhances ADCC for combination with mAb; chemotherapy seems to preferentially reduce regulatory T-cell function, enhancing the CpG-induced antitumour T-cell response
Asthma/allergy			
Monotherapy	Mouse: asthma, allergic rhinitis, conjunctivitis, allergic aspergillosis. Guinea pig: RSV sensitization Monkey: asthma. All reviewed in REF. 143	• AVE 7279 (Phase I; sanofi-aventis/Coley) • AVE 0675 (preclinical; sanofi-aventis/Coley) • 1018 ISS (Phase II; Dynavax) • IMO (preclinical; Novartis/Idera)	Suppress T_H2 response and IgE production[204]. Induce IDO expression, promoting anti-inflammatory Treg cells and reverse airway remodelling[144]. All reviewed in REF. 143
Vaccines	Mouse: asthma, allergy immunotherapy and atopic dermatitis	• B-class ODN 1018 ISS conjugated to protein[137,138] (Phase III; Dynavax)	Suppress or redirect T_H2 allergic response

ADCC, antibody dependent cellular cytotoxicity; DARPA, Defense Advanced Research Projects Agency; DC, dendritic cell; IDO, indoleamine (2,3)-dioxygenase; IP, intraperitoneally; mAb, monoclonal antibody; NHL, non-Hodgkins lymphoma; NIAID, National Institute of Allergy and Infectious Disease; NK, natural killer; NSCLC, non-small-cell lung cancer; ODN, oligodeoxynucleotides; RSV, respiratory syncytial virus.

might seem counterintuitive to combine this with TLR9 stimulation, and surprising that such combinations result in substantial improvements in survival in mouse tumour models using chemotherapy regimens ranging from the topoisomerase I inhibitor topotecan (Hycamtin; GlaxoSmithKline) to the alkylating agent cyclophosphamide and the antimetabolite 5-fluorouracil[148–150]. Where it has been tested, the increased antitumour efficacy of these combination approaches requires T cells but not natural killer cells, which is consistent with the hypothesis that *in vivo* activation of dendritic cells through TLR9 promotes an antitumour T-cell response that is capable of controlling the tumour and improving survival (FIG. 1).

Humans receiving certain chemotherapy regimens, such as taxanes, actually show *increased* T-cell and natural killer-cell immune competence[151], which might be related to induction of proinflammatory cytokine production, induction of homeostatic leukocyte proliferation, and reversal of the immune suppressive effects of regulatory T cells (Treg cells), which seem to protect the tumour against immune rejection[152].

On the basis of positive results in mouse tumour models, the effects of adding the B-class CpG ODN PF-3512676 (formerly called CPG 7909) to standard taxane/platinum chemotherapy for first-line treatment of stage IIIb/IV non-small-cell lung cancer (NSCLC) were investigated. In a Phase II randomized controlled human clinical trial, 112 chemotherapy-naive patients were randomized to receive four to six three-week cycles of standard chemotherapy alone or in combination with 0.2 mg per kg subcutaneous PF-3512676 on weeks two and three of each cycle. The primary endpoint for the trial — response rate (assessed by RECIST, using intention-to-treat analysis) — was significantly improved from 19% in the patients randomized to standard chemotherapy to 37% in the patients who also received PF-3512676[153]. The secondary endpoint of this trial, survival, showed a trend towards improvement from a median survival of 6.8 months in the chemotherapy arm to 12.8 months in the combination arm and an improvement in the 1-year survival from 33% to 50%[153]. As in the other clinical trials with TLR9 agonists, the most common side effects were

Box 5 | **Enhancing vaccines with TLR9 agonists**

A number of shortcomings of current vaccines have been enhanced by the addition of a Toll-like receptor 9 (TLR9) agonist in human trials or preclinical mouse models.

Vaccine deficiencies

- Need for several boosts to achieve protection
- Delay in rise of protective antibody titres
- Prevalence of vaccine non-responders, especially among immune-compromised populations
- Cost of antigen production
- Poorly protective antibody with low avidity
- Fall in antibody titre over time

Effect of TLR9 agonist

- Reduce number of vaccinations required to achieve seroprotection
- Accelerate seroconversion, possibly permitting post-exposure vaccination
- Reduce non-responder rate
- Reduce amount of antigen required
- Increase antibody avidity and protective activity
- More sustained antibody levels

mild to moderate injection-site reactions and transient flu-like symptoms. Grade 3 or 4 neutropaenia was more common in the combination arm, which is thought to reflect neutrophil redistribution, but febrile neutropaenia and grade 3/4 infections were actually slightly less common in the combination arm than in the chemotherapy alone arm. Thrombocytopaenia, a previously recognized phosphorothioate backbone effect that has occurred in all trials of antisense ODN, was seen more commonly in the combination arm, but there was no apparent increase in bleeding events. Based on these encouraging results, two controlled Phase III human clinical trials of PF-3512676 combined with doublet chemotherapy in first-line treatment of unresectable NSCLC were initiated by Pfizer in late 2005.

Safety of TLR9 activation in rodents and humans

In addition to the mechanism of action-related immune effects resulting from TLR9 activation, PS-ODN have a variety of sequence-independent backbone-related effects that have been characterized in detailed studies of antisense ODN[58,154,155]. PS-ODN are rapidly cleared from the circulation into the liver, kidneys and, to a lesser extent, the spleen and bone marrow[156,157]. Chronic dosing of PS-ODN in rodents results in a dose-dependent mononuclear cell infiltration in these organs, but such changes do not occur in monkeys or humans[58,158]. Hepatic effects specific to rodents include the activation of Kupffer cells with cellular hypertrophy and hyperplasia, basophilic granulation (thought to reflect PS-ODN deposition), and a mononuclear cell infiltrate in hepatic sinusoids and periportal regions[58,159]. In the kidneys, high local ODN concentrations reached after repeated high doses can induce degenerative lesions and necrosis in proximal tubules[58,160]. There have been no reports of adverse effects of PS-ODN on renal function in humans, despite the extensive clinical experience so far. Presumably these species-specific toxicities are a consequence of the cellular pattern of TLR9 expression, which determines the cytokines that will be produced in response to administration of a CpG ODN, and therefore the safety profile

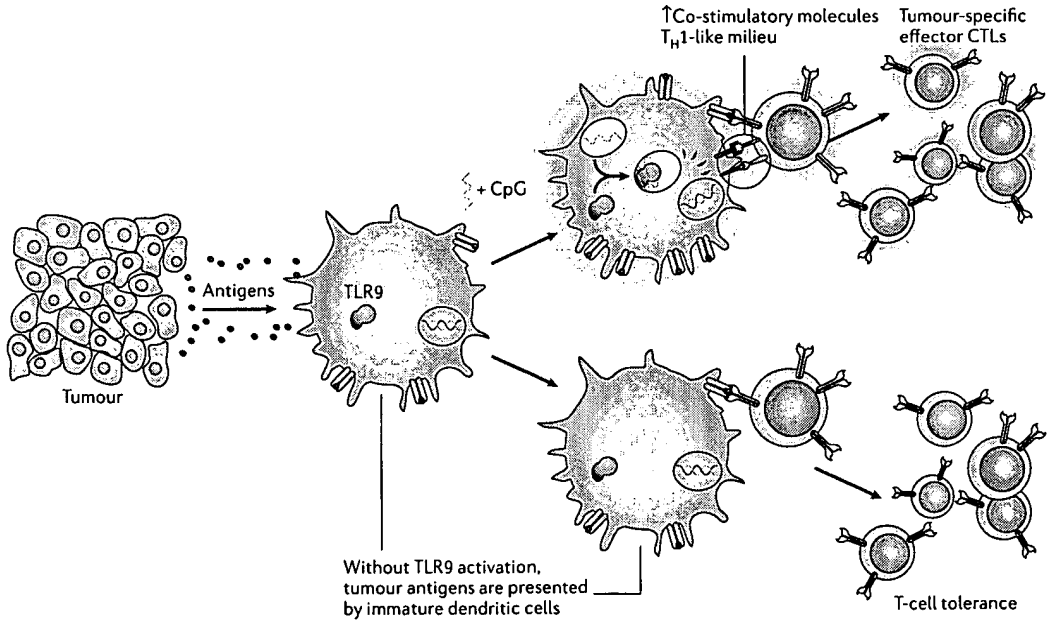


Figure 1 | **Switching on antitumour immunity by *in vivo* dendritic cell activation through TLR9.** In general, malignant tumours suppress immune function, and create an environment that favours the maintenance of T-cell tolerance, preventing the development of antitumour immunity. *In vivo* dendritic cell activation through Toll-like receptor 9 (TLR9) creates a T_H1-like cytokine and chemokine milieu and can up-regulate the expression of co-stimulatory molecules on the plasmacytoid dendritic cell (pDC), shifting T cells from tolerance, to a strong cytotoxic T-lymphocyte response against the tumour antigens.

of the drug. As TLR9 is expressed in a broader range of immune cells in rodents compared with primates, the rodent tends to over-predict toxicities that will occur in primates. For example, rodents respond to CpG ODN administration with high serum concentrations of pro-inflammatory cytokines such as TNFα, which can result in a lethal 'cytokine storm'[161], but in humans and primates there is no change in serum TNFα following CpG injection, which is generally well tolerated[59].

The major dose-limiting acute toxicity of PS-ODN in primates results from systemic activation of the alternative complement pathway with activation of leukocytes and changes in vascular permeability that can culminate in lethal cardiovascular collapse[162,163]. Fortunately, this toxicity does not occur below a threshold PS-ODN blood concentration of approximately 40–50 μg per ml, which typically is only reached when ODN is given via relatively rapid intravenous administration[58,162,164]. Inhibition of coagulation has been reported to result from binding of the PS-ODN to thrombin (specifically, to the tenase complex), and is reflected by prolongation of the activated partial thromboplastin time[58,165,166].

TLR9 activation by CpG ODN could also be proposed to induce adverse effects resulting from its mechanism of action. Early hypotheses that exposure to DNA containing CpG motifs generally induce autoimmunity[167] have proven unfounded. Nevertheless, CpG ODN treatment can clearly exacerbate autoimmunity in mouse models of lupus[168], multiple sclerosis[169], colitis[170] and arthritis[171]. Evolutionary considerations would suggest that the TLR9 pathway should not have evolved as an important immune defence mechanism unless its activation was controlled in some way, so as to prevent or at least limit the risk of inducing autoimmunity. Indeed, studies in various experimental models have established that TLR9 stimulation induces its own feedback suppression through mechanisms including induction of IFNα[172] or IFNγ secretion[173,174]; increased expression of IDO (which might promote development of immune-suppressive Treg cells)[144,172–176], cyclooxygenase-2 (COX2)[177] and suppressor of cytokine signalling, (SOCS); decreased expression of IRAK; and activation of ERK. There also seem to be some constitutively active pathways operating to limit the effects of TLR activation, such as single immunoglobulin IL-1R-related molecule (SIGIRR). The existence of these diverse counter-regulatory pathways that limit TLR9-induced immune activation suggests a potential to enhance the therapeutic efficacy of TLR9 agonists by co-administration of antagonists of one or more of these inhibitory pathways. Of course, such combinations might have a greater risk of inducing autoimmune disease. Understanding these mechanisms could make it possible to increase the therapeutic efficacy of CpG ODN by selectively disabling one or more of these counter-regulatory pathways, without inducing substantial added toxicity.

The safety profile of several TLR9 agonists in humans has been observed in the clinical trials described above over a more than 1,000-fold dose range from 0.0025–0.81 mg per kg. A maximal tolerated dose in humans has not been reported to date. The primary adverse events are dose-dependent local injection reactions (such as erythema, pain, swelling, induration, pruritus or warmth at the site of injection) or systemic flu-like reactions (such as headache, rigors, myalgia, pyrexia, nausea and vomiting), and are consistent with the known TLR9 agonist mechanism of action. Depending on the dose, systemic symptoms typically develop within 12–24 hours of dosing and persist for 1–2 days. At the low doses used in vaccine trials there seems to be a slight increase in the frequency of injection-site reactions, which are generally mild, above the frequency observed with the vaccine alone.

The clinical experience to date indicates that CpG ODN treatment of normal humans, cancer patients or individuals infected with HIV or HCV does not readily induce autoimmune disease. However, the duration of therapy has usually been less than 6 months; only a few patients have received chronic therapy with CpG ODN for longer than 3 years. In some animal models CpG ODN can even prevent autoimmune or inflammatory disease[172], but from a clinical perspective it might be prudent to consider the safety effects of CpG ODN in the same category as recombinant IFNα. Extensive clinical experience with IFNα has documented the induction of an autoimmune disease in 4–19% of chronically treated patients, and systemic lupus erythematosus (SLE) has been diagnosed in 0.15–0.7% of patients[178]. In most cases, such diseases resolve spontaneously after drug withdrawal. Based on the clinical experience to date, it seems that the incidence of autoimmunity and the overall toxicity will be lower with CpG ODN than has been observed with IFNα therapy; however, no definite conclusion on this can be reached until larger numbers of patients have been treated with CpG ODN for longer periods of time.

Box 6 | **Some unanswered questions and uncertainties in TLR9 biology**

- Is Toll-like receptor 9 (TLR9) expression regulated under physiological conditions, and by what stimuli?
- What cells express TLR9 in normal and disease states, and how does this explain the species-specific effects of CpG?
- What is the molecular basis for the different classes of CpG oligos?
- Does TLR9 directly and specifically bind CpG motifs?
- How does the interaction between TLR9 and the CpG motif activate signal transduction?
- Why can immune complexes containing mostly methylated vertebrate DNA activate immune cells, whereas vertebrate DNA alone does not?
- Could chronic TLR9 activation induce autoimmune disease in some people?
- What is the clinical significance of TLR9 polymorphisms, and how much heterogeneity is there in human responses to CpG ODN?

Future outlook

The CpG motif was described in 1995, and TLR9 was recognized to be the target of CpG DNA in 2000. Since then, half a dozen TLR9 agonists have been taken into human clinical trials, including three investigational products in Phase III trials, and there are already strong indications of substantial clinical benefit: it seems likely that the targeted activation of TLR9 using CpG ODN will enhance the treatment of cancer and infectious diseases, as well as offering new prospects for decreasing

the harmful inflammatory responses that characterize asthma and other allergic diseases. The rapidity of this clinical development and the breadth of the positive clinical data are impressive compared with the usual course of drug development against a novel target. The success of TLR9-based approaches has led to a resurgence of interest in the induction of therapeutic innate and adaptive immune responses. Although more studies are needed, and important questions remain to be addressed (BOX 6), the safety of these TLR9 agonists seems good.

A new direction in targeting TLR9 is suggested by recent studies implicating inappropriate activation of TLR9 by endogenous molecules in the pathogenesis of SLE and rheumatoid arthritis[179–183]. The results of these studies suggest that *antagonists* of TLR9 could be useful in the treatment of these autoimmune diseases, by blocking this inappropriate activation of B cells and pDC. Indeed, in mouse models, suppressive ODN designed to block TLR9 have already shown benefit in preventing or reversing both SLE and rheumatoid arthritis[54,184,185]. TLR9 could turn out to be a target for which both agonists and antagonists could find therapeutic application, depending on the clinical setting. The coming years and a lot of work should provide the answer to this question.

1. Iwasaki, A. & Medzhitov, R. Toll-like receptor control of the adaptive immune responses. *Nature Immunol.* **5**, 987–995 (2004).
2. Barton, G. M., Kagan, J. C. & Medzhitov, R. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. *Nature Immunol.* **7**, 49–56 (2006).
This report demonstrates that the ability of TLR9 to detect specifically viral but not self DNA is a consequence of TLR9's unusual intracellular localization.
3. Ishii, K. J. *et al.* A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. *Nature Immunol.* **7**, 40–48 (2006).
4. Okabe, Y., Kawane, K., Akira, S., Taniguchi, T. & Nagata, S. Toll-like receptor-independent gene induction program activated by mammalian DNA escaped from apoptotic DNA degradation. *J. Exp. Med.* **202**, 1333–1339 (2005).
5. Liu, Y. J. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu. Rev. Immunol.* **23**, 275–306 (2005).
6. Hayashi, F., Means, T. K. & Luster, A. D. Toll-like receptors stimulate human neutrophil function. *Blood* **102**, 2660–2669 (2003).
7. Li, J. *et al.* CpG DNA-mediated immune response in pulmonary endothelial cells. *Am. J. Physiol. Lung Cell Mol. Physiol.* **287**, L552-L558 (2004).
8. Platz, J. *et al.* Microbial DNA induces a host defense reaction of human respiratory epithelial cells. *J. Immunol.* **173**, 1219–1223 (2004).
9. Asselin-Paturel, C. *et al.* Type I interferon dependence of plasmacytoid dendritic cell activation and migration. *J. Exp. Med.* **201**, 1157–1167 (2005).
10. Krieg, A. M. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* **20**, 709–760 (2002).
11. Jung, J. *et al.* Distinct response of human B cell subpopulations in recognition of an innate immune signal, CpG DNA. *J. Immunol.* **169**, 2368–2373 (2002).
12. Bernasconi, N. L., Traggiai, E. & Lanzavecchia, A. Maintenance of serological memory by polyclonal activation of human memory B cells. *Science* **298**, 2199–2202 (2002).
13. Bernasconi, N. L., Onai, N. & Lanzavecchia, A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. *Blood* **101**, 4500–4504 (2003).
14. Traggiai, E. *et al.* An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. *Nature Med.* **10**, 871–875 (2004).
Building on their earlier studies (references 12 and 13) into the ability of CpG ODN to activate B cells and cooperate with B cell antigen receptor signaling, this paper reports a remarkably efficient method for generating human monoclonal antibodies.
15. Poeck, H. *et al.* Plasmacytoid dendritic cells, antigen and CpG-C license human B cells for plasma cell differentiation and immunoglobulin production in the absence of T cell help. *Blood* **103**, 3058–3064 (2004).
16. Krieg, A. M. *et al.* CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549 (1995).
17. Yi, A. K. *et al.* CpG motifs in bacterial DNA activate leukocytes through the pH-dependent generation of reactive oxygen species. *J. Immunol.* **160**, 4755–4761 (1998).
18. Ahmad-Nejad, P. *et al.* Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. *Eur. J. Immunol.* **32**, 1958–1968 (2002).
19. Hacker, H. *et al.* CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J.* **17**, 6230–6240 (1998).
20. Manzel, L., Strekowski, L., Ismail, F. M., Smith, J. C. & Macfarlane, D. E. Antagonism of immunostimulatory CpG-oligodeoxynucleotides by 4-aminoquinolines and other weak bases: mechanistic studies. *J Pharmacol. Exp. Ther.* **291**, 1337–1347 (1999).
21. Ishii, K. J. *et al.* Potential role of phosphatidylinositol 3 kinase, rather than DNA-dependent protein kinase, in CpG DNA-induced immune activation. *J. Exp. Med.* **196**, 269–274 (2002).
22. Hacker, H. *et al.* Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor-associated factor (TRAF)6. *J. Exp. Med.* **192**, 595–600 (2000).
23. Schnare, M., Holtdagger, A. C., Takeda, K., Akira, S. & Medzhitov, R. Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. *Curr. Biol.* **10**, 1139–1142 (2000).
24. Muzio, M., Ni, J., Feng, P. & Dixit, V. M. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science* **278**, 1612–1615 (1997).
25. Muzio, M., Natoli, G., Saccani, S., Levrero, M. & Mantovani, A. The human toll signaling pathway: divergence of nuclear factor κB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). *J. Exp. Med.* **187**, 2097–2101 (1998).
26. Rutz, M. *et al.* Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner. *Eur. J. Immunol.* **34**, 2541–2550 (2004).
Direct binding of TLR9 to CpG ODN has been reported by several groups but is still poorly understood. These investigators report binding to be at least partly sequence-specific under the low-pH conditions present in the endosome.
27. Hartmann, G. & Krieg, A. M. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J. Immunol.* **164**, 944–953 (2000).
28. Yi, A. K. & Krieg, A. M. Rapid induction of mitogen-activated protein kinases by immune stimulatory CpG DNA. *J. Immunol.* **161**, 4493–4497 (1998).
29. Takeshita, F. & Klinman, D. M. CpG ODN-mediated regulation of IL-12 p40 transcription. *Eur. J. Immunol.* **30**, 1967–1976 (2000).
30. Tsujimura, H. *et al.* Toll-like receptor 9 signaling activates NF-κB through IFN regulatory factor-8/IFN consensus sequence binding protein in dendritic cells. *J. Immunol.* **172**, 6820–6827 (2004).
31. Choudhury, B. K. *et al.* *In vivo* role of p38 mitogen-activated protein kinase in mediating the anti-inflammatory effects of CpG oligodeoxynucleotide in murine asthma. *J. Immunol.* **169**, 5955–5961 (2002).
32. Yi, A. K., Yoon, J. G. & Krieg, A. M. Convergence of CpG DNA- and BCR-mediated signals at the c-Jun N- terminal kinase and NF-κB activation pathways: regulation by mitogen-activated protein kinases. *Int. Immunol.* **15**, 577–591 (2003).
33. Yi, A. K. *et al.* Role of mitogen-activated protein kinases in CpG DNA-mediated IL-10 and IL-12 production: central role of extracellular signal-regulated kinase in the negative feedback loop of the CpG DNA-mediated Th1 response. *J. Immunol.* **168**, 4711–4720 (2002).
34. Rankin, R. *et al.* CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved. *Antisense Nucleic Acid Drug Dev.* **11**, 333–340 (2001).
35. Yi, A. K., Chang, M., Peckham, D. W., Krieg, A. M. & Ashman, R. F. CpG oligodeoxyribonucleotides rescue mature spleen B cells from spontaneous apoptosis and promote cell cycle entry. *J. Immunol.* **160**, 5898–5906 (1998).
36. Bauer, S. *et al.* Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc. Natl Acad. Sci. USA* **98**, 9237–9242 (2001).
The first paper showing a direct and species-specific interaction between TLR9 and different CpG motifs.
37. Latz, E. *et al.* TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nature Immunol.* **5**, 190–198 (2004).
A major advance in providing the clearest understanding yet into the intracellular trafficking of TLR9, and its response to CpG ODN.
38. Ballas, Z. K., Rasmussen, W. L. & Krieg, A. M. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* **157**, 1840–1845 (1996).
39. Hartmann, G. *et al.* Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses *in vitro* and *in vivo*. *J. Immunol.* **164**, 1617–1624 (2000).
40. Pisetsky, D. S. & Reich, C. F., III. The influence of base sequence on the immunological properties of defined oligonucleotides. *Immunopharmacology* **40**, 199–208 (1998).
41. Roberts, T. L., Sweet, M. J., Hume, D. A. & Stacey, K. J. Cutting edge: species-specific TLR9-mediated recognition of CpG and non-CpG phosphorothioate-modified oligonucleotides. *J. Immunol.* **174**, 605–608 (2005).
42. Vollmer, J. *et al.* Oligodeoxynucleotides lacking CpG dinucleotides mediate Toll-like receptor 9 dependent T helper type 2 biased immune stimulation. *Immunology* **113**, 212–223 (2004).
TLR9 can respond to more than CpG; this paper demonstrates that some CpG-free ODN can activate TLR9, but induce a distinct profile of cytokine production, revealing an unexpected plasticity in TLR9 biology.
43. Vollmer, J. *et al.* Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. *Eur. J. Immunol.* **34**, 251–262 (2004).
Together with references 196 and 197 these three papers define the third class of CpG ODN, based on unique structural characteristics.
44. Hemmi, H., Kaisho, T., Takeda, K. & Akira, S. The roles of Toll-like receptor 9, MyD88, and DNA-dependent protein kinase catalytic subunit in the effects of two distinct CpG DNAs on dendritic cell subsets. *J. Immunol.* **170**, 3059–3064 (2003).
45. Honda, K. *et al.* Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. *Nature* **434**, 1035–1040 (2005).

This paper provides intriguing evidence that ODN structures with different biological properties have distinct intracellular distribution.
46. Uhlmann, E. & Vollmer, J. Recent advances in the development of immunostimulatory oligonucleotides. *Curr. Opin. Drug Discov. Devel.* **6**, 204–217 (2003).
47. Kandimalla, E. R., Zhu, F. G., Bhagat, L., Yu, D. & Agrawal, S. Toll-like receptor 9: modulation of recognition and cytokine induction by novel synthetic CpG DNAs. *Biochem. Soc. Trans.* **31**, 654–658 (2003).
48. Krieg, A. M., Guga, P. & Stec, W. P-chirality-dependent immune activation by phosphorothioate CpG oligodeoxynucleotides. *Oligonucleotides* **13**, 491–499 (2003).
49. Krieg, A. M. *et al.* Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs. *Proc. Natl Acad. Sci. USA* **95**, 12631–12636 (1998).
50. Yamada, H. *et al.* Effect of suppressive DNA on CpG-induced immune activation. *J. Immunol.* **169**, 5590–5594 (2002).
51. Lenert, P., Stunz, L. L., Yi, A. K., Krieg, A. M. & Ashman, R. F. CpG stimulation of primary mouse B cells is blocked by inhibitory oligodeoxyribonucleotide s at a site proximal to NF-kB activation. *Antisense Nucleic Acid Drug Dev.* **11**, 247–256 (2001).
52. Jurk, M. Selective inhibition of Toll-like receptor-mediated signalling by inhibitory oligodeoxynucleotides. *Clin. Invest. Med.* **27**, 2333 (2005).
53. Beignon, A. S. *et al.* Endocytosis of HIV-1 activates plasmacytoid dendritic cells via Toll-like receptor-viral RNA interactions. *J. Clin. Invest.* **15**, 3265–3275 (2005).
54. Barrat, F. J. *et al.* Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. *J. Exp. Med.* **202**, 1131–1139 (2005).
55. Shirota, H., Gursel, M. & Klinman, D. M. Suppressive oligodeoxynucleotides inhibit Th1 differentiation by blocking IFN-γ- and IL-12-mediated signaling. *J. Immunol.* **173**, 5002–5007 (2004).
56. Lenert, P., Rasmussen, W., Ashman, R. F. & Ballas, Z. K. Structural characterization of the inhibitory DNA motif for the type A (D)-CpG-induced cytokine secretion and NK-cell lytic activity in mouse spleen cells. *DNA Cell Biol.* **22**, 621–631 (2003).
57. Geary, R. S. *et al.* Pharmacokinetics and metabolism in mice of a phosphorothioate oligonucleotide antisense inhibitor of C-raf-1 kinase expression. *Drug Metab. Dispos.* **25**, 1272–1281 (1997).
58. Levin, A. A., Henry, S. & Monteith, D. *Antisense Drug Technology* (ed. Crooke, S. T.) 201–267 (Marcel Dekker, New York, 2001).
59. Krieg, A. M., Efler, S. M., Wittpoth, M., Al Adhami, M. J. & Davis, H. L. Induction of systemic TH1-like innate immunity in normal volunteers following subcutaneous but not intravenous administration of CPG 7909, a synthetic B-class CpG oligodeoxynucleotide TLR9 agonist. *J. Immunother.* **27**, 460–471 (2004).
60. Elkins, K. L., Rhinehart-Jones, T. R., Stibitz, S., Conover, J. S. & Klinman, D. M. Bacterial DNA containing CpG motifs stimulates lymphocyte-dependent protection of mice against lethal infection with intracellular bacteria. *J. Immunol.* **162**, 2291–2298 (1999).
61. Gramzinski, R. A. *et al.* Interleukin-12- and γinterferon-dependent protection against malaria conferred by CpG oligodeoxynucleotide in mice. *Infect. Immun.* **69**, 1643–1649 (2001).
62. Zimmermann, S. *et al.* CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. *J. Immunol.* **160**, 3627–3630 (1998).
63. Krieg, A. M., Love-Homan, L., Yi, A. K. & Harty, J. T. CpG DNA induces sustained IL-12 expression *in vivo* and resistance to *Listeria monocytogenes* challenge. *J. Immunol.* **161**, 2428–2434 (1998).
64. Klinman, D. M., Conover, J. & Coban, C. Repeated administration of synthetic oligodeoxynucleotides expressing CpG motifs provides long-term protection against bacterial infection. *Infect. Immun.* **67**, 5658–5663 (1999).
65. Klinman, D. M., Verthelyi, D., Takeshita, F. & Ishii, K. J. Immune recognition of foreign DNA: a cure for bioterrorism? *Immunity* **11**, 123–129 (1999).
66. Rees, D. G. *et al.* CpG-DNA protects against a lethal orthopoxvirus infection in a murine model. *Antiviral Res.* **65**, 87–95 (2005).
67. Deng, J. C. *et al.* CpG oligodeoxynucleotides stimulate protective innate immunity against pulmonary Klebsiella infection. *J. Immunol.* **173**, 5148–5155 (2004).
68. Ray, N. B. & Krieg, A. M. Oral pretreatment of mice with CpG DNA reduces susceptibility to oral or intraperitoneal challenge with virulent Listeria monocytogenes. *Infect. Immun.* **71**, 4398–4404 (2003).
69. Klinman, D. M. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nature Rev. Immunol.* **4**, 249–259 (2004).
70. Weighardt, H. *et al.* Increased resistance against acute polymicrobial sepsis in mice challenged with immunostimulatory CpG oligodeoxynucleotides is related to an enhanced innate effector cell response. *J. Immunol.* **165**, 4537–4543 (2000).
71. Pyles, R. B. *et al.* Use of immunostimulatory sequence-containing oligonucleotides as topical therapy for genital herpes simplex virus type 2 infection. *J. Virol.* ***76, 11387–11396*** (2002).
72. Ashkar, A. A., Bauer, S., Mitchell, W. J., Vieira, J. & Rosenthal, K. L. Local delivery of CpG oligodeoxynucleotides induces rapid changes in the genital mucosa and inhibits replication, but not entry, of herpes simplex virus type 2. *J. Virol.* **77**, 8948–8956 (2003).
73. Walker, P. S. *et al.* Immunostimulatory oligodeoxynucleotides promote protective immunity and provide systemic therapy for leishmaniasis via IL-12- and IFN-γ-dependent mechanisms. *Proc. Natl Acad. Sci. USA* **96**, 6970–6975 (1999).
74. Cho, J. Y. *et al.* Immunostimulatory DNA sequences inhibit respiratory syncytial viral load, airway inflammation, and mucus secretion. *J. Allergy Clin. Immunol.* **108**, 697–702 (2001).
75. Juffermans, N. P. *et al.* CpG oligodeoxynucleotides enhance host defense during murine tuberculosis. *Infect. Immun.* **70**, 147–152 (2002).
76. Olbrich, A. R. *et al.* Effective postexposure treatment of retrovirus-induced disease with immunostimulatory DNA containing CpG motifs. *J. Virol.* **76**, 11397–11404 (2002).
77. Freidag, B. L. *et al.* CpG oligodeoxynucleotides and interleukin-12 improve the efficacy of Mycobacterium bovis BCG vaccination in mice challenged with M. tuberculosis. *Infect. Immun.* **68**, 2948–2953 (2000).
78. Ramirez-Pineda, J. R., Frohlich, A., Berberich, C. & Moll, H. Dendritic cells (DC) activated by CpG DNA *ex vivo* are potent inducers of host resistance to an intracellular pathogen that is independent of IL-12 derived from the immunizing DC. *J. Immunol.* **172**, 6281–6289 (2004).
79. Ishii, K. J. *et al.* CpG-activated Thy1.2+ dendritic cells protect against lethal Listeria monocytogenes infection. *Eur. J. Immunol.* **35**, 2397–2405 (2005).
80. Lugo-Villarino, G., Ito, S., Klinman, D. M. & Glimcher, L. H. The adjuvant activity of CpG DNA requires T-bet expression in dendritic cells. *Proc. Natl Acad. Sci. USA* **102**, 13248–13253 (2005).
81. Sajic, D. *et al.* Parameters of CpG oligodeoxy-nucleotide-induced protection against intravaginal HSV-2 challenge. *J Med. Virol.* **71**, 561–568 (2003).
82. Isogawa, M., Robek, M. D., Furuichi, Y. & Chisari, F. V. Toll-like receptor signaling inhibits hepatitis B virus replication *in vivo*. *J. Virol.* **79**, 7269–7272 (2005).
83. Verthelyi, D. *et al.* CpG oligodeoxynucleotides protect normal and SIV-infected macaques from Leishmania infection. *J. Immunol.* **170**, 4717–4723 (2003).
84. Rehermann, B. & Nascimbeni, M. Immunology of hepatitis B virus and hepatitis C virus infection. *Nature Rev. Immunol.* **5**, 215–229 (2005).
85. McHutchison, J. G. *et al.* Relationships of HCV RNA responses to CPG 10101, a TLR9 agonist: pharmacodynamics & patient characteristics. *Hepatology* **42**, 249A (2005).
86. Olbrich, A. R., Schimmer, S. & Dittmer, U. Preinfection treatment of resistant mice with CpG oligodeoxynucleotides renders them susceptible to friend retrovirus-induced leukemia. *J. Virol.* **77**, 10658–10662 (2003).
87. Ito, S., Pedras-Vasconcelos, J. & Klinman, D. M. CpG oligodeoxynucleotides increase the susceptibility of normal mice to infection by Candida albicans. *Infect. Immun.* **73**, 6154–6156 (2005).
88. Equils, O. *et al.* Toll-like receptor 2 (TLR2) and TLR9 signaling results in HIV-Long terminal repeat trans-activation and HIV replication in HIV-1 transgenic mouse spleen cells: implications of simultaneous activation of TLRs on HIV replication. *J. Immunol.* **170**, 5159–5164 (2003).
89. Agrawal, S. and Martin, R. R. Was induction of HIV1 through TLR9? *J. Immunol.* **171**, 1621 (2003).
90. Gurney, K. B., Colantonio, A. D., Blom, B., Spits, H. & Uittenbogaart, C. H. Endogenous IFN-α production by plasmacytoid dendritic cells exerts an antiviral effect on thymic HIV-1 infection. *J. Immunol.* **173**, 7269–7276 (2004).
91. Schlaepfer, E. *et al.* CpG oligodeoxynucleotides block human immunodeficiency virus type 1 replication in human lymphoid tissue infected *ex vivo*. *J. Virol.* **78**, 12344–12354 (2004).
92. Saez, R., Echaniz, P., de Juan, M. D., Iribarren, J. A. & Cuadrado, E. HIV-infected progressors and long-term non-progressors differ in their capacity to respond to an A-class CpG oligodeoxynucleotide. *AIDS* **19**, 1924–1925 (2005).
93. Cooper, C. L. *et al.* CPG 7909 adjuvant improves hepatitis B virus vaccine seroprotection in antiretroviral-treated HIV-infected adults. *AIDS* **19**, 1473–1479 (2005).
In this clinical trial a TLR9 agonist was shown to have strong vaccine adjuvant activity even in immune-compromised (HIV-infected) humans, extending the results of vaccine trials in normal volunteers (references 129 and 130).
94. Davis, H. L. *et al.* CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J. Immunol.* **160**, 870–876 (1998).
95. Liu, N., Ohnishi, N., Ni, L., Akira, S. & Bacon, K. B. CpG directly induces T-bet expression and inhibits IgG1 and IgE switching in B cells. *Nature Immunol.* **4**, 687–693 (2003).
96. He, B., Qiao, X. & Cerutti, A. CpG DNA induces IgG class switch DNA recombination by activating human B cells through an innate pathway that requires TLR9 and cooperates with IL-10. *J. Immunol.* **173**, 4479–4491 (2004).
97. Lipford, G. B., Sparwasser, T., Zimmermann, S., Heeg, K. & Wagner, H. CpG-DNA-mediated transient lymphadenopathy is associated with a state of Th1 predisposition to antigen-driven responses. *J. Immunol.* **165**, 1228–1235 (2000).
98. Sparwasser, T., Vabulas, R. M., Villmow, B., Lipford, G. B. & Wagner, H. Bacterial CpG-DNA activates dendritic cells *in vivo*: T helper cell-independent cytotoxic T cell responses to soluble proteins. *Eur. J. Immunol.* **30**, 3591–3597 (2000).
99. Tighe, H. *et al.* Conjugation of protein to immunostimulatory DNA results in a rapid, long-lasting and potent induction of cell-mediated and humoral immunity. *Eur. J. Immunol.* **30**, 1939–1947 (2000).
100. Hartmann, E. *et al.* Identification and functional analysis of tumor-infiltrating plasmacytoid dendritic cells in head and neck cancer. *Cancer Res.* **63**, 6478–6487 (2003).
This report shows that CpG responses are severely suppressed in dendritic cells isolated from primary human tumours, but less so in the draining lymph nodes.
101. Wettstein, P. J., Borson, N. D., Park, J. G., McNallan, K. T. & Reed, A. M. Cysteine-tailed class I-binding peptides bind to CpG adjuvant and enhance primary CTL responses. *J. Immunol.* **175**, 3681–3689 (2005).
102. Kim, S. K. *et al.* Comparison of the effect of different immunological adjuvants on the antibody and T-cell response to immunization with MUC1-KLH and GD3-KLH conjugate cancer vaccines. *Vaccine* **18**, 597–603 (2000).
103. Chu, R. S., Targoni, O. S., Krieg, A. M., Lehmann, P. V. & Harding, C. V. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J. Exp. Med.* **186**, 1623–1631 (1997).
104. Lipford, G. B. *et al.* CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. *Eur. J. Immunol.* **27**, 2340–2344 (1997).
105. Roman, M. *et al.* Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants [see comments]. *Nature Med.* **3**, 849–854 (1997).
106. Weeratna, R. D., McCluskie, M. J., Xu, Y. & Davis, H. L. CpG DNA induces stronger immune responses with less toxicity than other adjuvants. *Vaccine* **18**, 1755–1762 (2000).
107. Sugai, T. *et al.* A CpG-containing oligodeoxynucleotide as an efficient adjuvant counterbalancing the Th1/Th2 immune response in diphtheria-tetanus-pertussis vaccine. *Vaccine* **23**, 5450–5456 (2005).

108. Oumouna, M., Mapletoft, J. W., Karvonen, B. C., Babiuk, L. A. & van Drunen Littel-van den Hurk. Formulation with CpG oligodeoxynucleotides prevents induction of pulmonary immunopathology following priming with formalin-inactivated or commercial killed bovine respiratory syncytial virus vaccine. *J. Virol.* **79**, 2024–2032 (2005).
109. Brazolot Millan, C. L., Weeratna, R., Krieg, A. M., Siegrist, C. A. & Davis, H. L. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. *Proc. Natl Acad. Sci. USA* **95**, 15553–15558 (1998).
110. Weeratna, R. D., Brazolot Millan, C. L., McCluskie, M. J., Siegrist, C. A. & Davis, H. L. Priming of immune responses to hepatitis B surface antigen in young mice immunized in the presence of maternally derived antibodies. *FEMS Immunol. Med. Microbiol.* **30**, 241–247 (2001).
111. Schirmbeck, R. & Reimann, J. Modulation of gene-gun-mediated Th2 immunity to hepatitis B surface antigen by bacterial CpG motifs or IL-12. *Intervirology* **44**, 115–123 (2001).
112. Zhou, X., Zheng, L., Liu, L., Xiang, L. & Yuan, Z. T helper 2 immunity to hepatitis B surface antigen primed by gene-gun-mediated DNA vaccination can be shifted towards T helper 1 immunity by codelivery of CpG motif-containing oligodeoxynucleotides. *Scand. J. Immunol.* **58**, 350–357 (2003).
113. Weeratna, R. D., Brazolot Millan, C. L., McCluskie, M. J. & Davis, H. L. CpG ODN can re-direct the Th bias of established Th2 immune responses in adult and young mice. *FEMS Immunol. Med. Microbiol.* **32**, 65–71 (2001).
114. Manning, B. M., Enioutina, E. Y., Visic, D. M., Knudson, A. D. & Daynes, R. A. CpG DNA functions as an effective adjuvant for the induction of immune responses in aged mice. *Exp. Gerontol.* **37**, 107–126 (2001).
115. Maletto, B., Ropolo, A., Moron, V. & Pistoresi-Palencia, M. C. CpG-DNA stimulates cellular and humoral immunity and promotes Th1 differentiation in aged BALB/c mice. *J. Leukoc. Biol.* **72**, 447–454 (2002).
116. Alignani, D. *et al.* Orally administered OVA/CpG-ODN induces specific mucosal and systemic immune response in young and aged mice. *J. Leukoc. Biol.* **77**, 898–905 2005.
117. Krieg, A. M. & Davis, H. L. Enhancing vaccines with immune stimulatory CpG DNA. *Curr. Opin. Mol. Ther.* **3**, 15–24 (2001).
118. Moldoveanu, Z., Love-Homan, L., Huang, W. Q. & Krieg, A. M. CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. *Vaccine* **16**, 1216–1224 (1998).
119. Gallichan, W. S. *et al.* Intranasal immunization with CpG oligodeoxynucleotides as an adjuvant dramatically increases IgA and protection against herpes simplex virus- 2 in the genital tract. *J. Immunol.* **166**, 3451–3457 (2001).
120. McCluskie, M. J. & Davis, H. L. CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. *J. Immunol.* **161**, 4463–4466 (1998).
121. McCluskie, M. J. & Davis, H. L. Oral, intrarectal and intranasal immunizations using CpG and non-CpG oligodeoxynucleotides as adjuvants. *Vaccine* **19**, 413–422 (2001).
122. Kwant, A. & Rosenthal, K. L. Intravaginal immunization with viral subunit protein plus CpG oligodeoxynucleotides induces protective immunity against HSV-2. *Vaccine* **22**, 3098–3104 (2004).
123. Eastcott, J. W. *et al.* Oligonucleotide containing CpG motifs enhances immune response to mucosally or systemically administered tetanus toxoid. *Vaccine* **19**, 1636–1642 (2001).
124. McCluskie, M. J., Weeratna, R. D., Krieg, A. M. & Davis, H. L. CpG DNA is an effective oral adjuvant to protein antigens in mice. *Vaccine* **19**, 950–957 (2001).
125. Dong, J. L., Liang, B. G., Jin, Y. S., Zhang, W. J. & Wang, T. Oral immunization with pBsVP6-transgenic alfalfa protects mice against rotavirus infection. *Virology* **339**, 153–163 (2005).
126. Nesburn, A. B. *et al.* Local and systemic B cell and Th1 responses induced following ocular mucosal delivery of multiple epitopes of herpes simplex virus type 1 glycoprotein D together with cytosine-phosphate-guanine adjuvant. *Vaccine* **23**, 873–883 (2005).
127. Berry, L. J. *et al.* Transcutaneous immunization with combined cholera toxin and CpG adjuvant protects against Chlamydia muridarum genital tract infection. *Infect. Immun.* **72**, 1019–1028 (2004).
128. Dumais, N., Patrick, A., Moss, R. B., Davis, H. L. & Rosenthal, K. L. Mucosal immunization with inactivated human immunodeficiency virus plus CpG oligodeoxynucleotides induces genital immune responses and protection against intravaginal challenge. *J. Infect. Dis.* **186**, 1098–1105 (2002).
129. Cooper, C. L. *et al.* CpG 7909, an immunostimulatory TLR9 agonist oligodeoxynucleotide, as adjuvant to Engerix-B HBV vaccine in healthy adults: a double-blind Phase I/II study. *J. Clin. Immunol.* **24**, 693–702 (2004).
130. Halperin, S. A. *et al.* A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant. *Vaccine* **21**, 2461–2467 (2003).
References 129 and 130 show dramatic adjuvant activity of two different B-Class CpG ODN added to the hepatitis B surface antigen both alone and in combination with alum.
131. Siegrist, C. A. *et al.* Co-administration of CpG oligonucleotides enhances the late affinity maturation process of human anti-hepatitis B vaccine response. *Vaccine* **23**, 615–622 (2004).
132. Rynkiewicz, D. *et al.* Marked enhancement of antibody response to anthrax vaccine adsorbed with CPG 7909 in healthy volunteers. *Intersci. Conf. Antimicrob. Agents Chemother.* Poster (2005).
133. Weeratna, R., Comanita, L. & Davis, H. L. CPG ODN allows lower dose of antigen against hepatitis B surface antigen in BALB/c mice. *Immunol. Cell Biol.* **81**, 59–62 (2003).
134. Cooper, C. L. *et al.* Safety and Immunogenicity of CpG 7909 Injection as an Adjuvant to Fluarix Influenza Vaccine. *Vaccine* **22**, 3136–3143 (2004).
135. Kline, J. N. *et al.* Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J. Immunol.* **160**, 2555–2559 (1998).
136. Jain, V. V. *et al.* CpG-oligodeoxynucleotides inhibit airway remodeling in a murine model of chronic asthma. *J. Allergy Clin. Immunol.* **110**, 867–872 (2002).
137. Creticos, P. S., Eiden, J. J. & *et al.* Immunotherapy with immunostimulatory oligonucleotides linked to purified ragweed Amb a 1 allergen: effects on antibody production, nasal allergen provocation, and ragweed seasonal rhinitis. *J. Allergy Clin. Immunol.* **109**, 742–743 (2002).
138. Simons, F. E., Shikishima, Y., Van Nest, G., Eiden, J. J. & HayGlass, K. T. Selective immune redirection in humans with ragweed allergy by injecting Amb a 1 linked to immunostimulatory DNA. *J. Allergy Clin. Immunol.* **113**, 1144–1151 (2004).
This clinical trial report demonstrates that the anti-allergic effects of CpG ODN are not limited to mice, but are also seen in humans.
139. van Ojik, H. *et al.* Phase I/II study with CpG 7909 as adjuvant to vaccination with MAGA-3 protein in patients with MAGE-3 positive tumors. *Ann. Oncol.* **13**, 157 (2002).
140. Speiser, D. E. *et al.* Rapid and strong human CD8(+) T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. *J. Clin. Invest.* **115**, 739–746 (2005).
This is the first human clinical trial report of a CpG ODN added to a cancer vaccine, and shows that patients receiving the vaccine made a strong CD8 T-cell response to the tumour antigen.
141. Jain, V. V. *et al.* Mucosal immunotherapy with CpG oligodeoxynucleotides reverses a murine model of chronic asthma induced by repeated antigen exposure. *Am. J. Physiol. Lung Cell Mol. Physiol.* 285, L1137-L1146 (2003).
142. Fanucchi, M. V. *et al.* Immunostimulatory oligonucleotides attenuate airways remodelling in allergic monkeys. *Am. J. Respir. Crit. Care Med.* **170**, 1153–1157 (2004).
143. Racila, D. M. & Kline, J. N. Perspectives in asthma: molecular use of microbial products in asthma prevention and treatment. *J. Allergy Clin. Immunol.* **116**, 1202–1205 (2005).
144. Hayashi, T. *et al.* Inhibition of experimental asthma by indoleamine 2, 3-dioxygenase. *J. Clin. Invest.* **114**, 270–279 (2004).
This detailed investigation into the mechanism of action of a CpG ODN in treating experimental asthma in a mouse model revealed new insights into the surprising anti-inflammatory role of TLR9 activation in the lung. See also references 175 and 176.
145. Krieg, A. M. Antitumor applications of stimulating Toll-like receptor 9 with CpG oligodeoxynucleotides. *Curr. Oncol. Rep.* **6**, 88–95 (2004).
146. Link, B. *et al.* Oligodeoxynucleotide CPG 7909 Delivered as intravenous infusion demonstrates immunologic modulation in patients with previously treated non-Hodgkin's lymphoma. *J. Immunother.* (in the press).
147. Friedberg, J. W. *et al.* Combination immunotherapy with a CpG oligonucleotide (1018 ISS) and rituximab in patients with non-Hodgkin lymphoma: increased interferon-α/β-inducible gene expression, without significant toxicity. *Blood* **105**, 489–495 (2005).
148. Weigel, B. J., Rodeberg, D. A., Krieg, A. M. & Blazar, B. R. CpG oligodeoxynucleotides potentiate the antitumor effects of chemotherapy or tumor resection in an orthotopic murine model of rhabdomyosarcoma. *Clin. Cancer Res.* **9**, 3105–3114 (2003).
149. Balsari, A. *et al.* Combination of a CpG-oligodeoxynucleotide and a topoisomerase I inhibitor in the therapy of human tumour xenografts. *Eur. J. Cancer* **40**, 1275–1281 (2004).
150. Wang, X. S., Sheng, Z., Ruan, Y. B., Guang, Y. & Yang, M. L. CpG oligodeoxynucleotides inhibit tumor growth and reverse the immunosuppression caused by the therapy with 5-fluorouracil in murine hepatoma. *World J. Gastroenterol.* **11**, 1220–1224 (2005).
151. Carson, W. E., III, Shapiro, C. L., Crespin, T. R., Thornton, L. M. & Andersen, B. L. Cellular immunity in breast cancer patients completing taxane treatment. *Clin. Cancer Res.* **10**, 3401–3409 (2004).
152. Emens, L. A., Reilly, R. T. & Jaffee, E. M. Augmenting the potency of breast cancer vaccines: combined modality immunotherapy. *Breast Dis.* **20**, 13–24 (2004).
153. Manegold, C., Leichman, G., Gravenor, D. & *et al.* Addition of PF-3512676 (CpG 7909) to a Taxane/Platinum regimen for first-line treatment of unresectable non-small cell lung cancer (NSCLC) improves objective response — Phase II clinical trial. *Eur. J. Cancer* **3** (Suppl.), 326 A1131 (2005).
154. Levin, A. A. A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. *Biochim. Biophys. Acta* **1489**, 69–84 (1999).
155. Monteith, D. K. & Levin, A. A. Synthetic oligonucleotides: the development of antisense therapeutics. *Toxicol. Pathol.* **27**, 8–13 (1999).
156. Geary, R. S., Leeds, J. M., Henry, S. P., Monteith, D. K. & Levin, A. A. Antisense oligonucleotide inhibitors for the treatment of cancer: 1. Pharmacokinetic properties of phosphorothioate oligodeoxynucleotides. *Anticancer Drug Des.* **12**, 383–393 (1997).
157. Cossum, P. A. *et al.* Disposition of the 14C-labeled phosphorothioate oligonucleotide ISIS 2105 after intravenous administration to rats. *J. Pharmacol. Exp. Ther.* **267**, 1181–1190 (1993).
158. Henry, S. P., Taylor, J., Midgley, L., Levin, A. A. & Kornbrust, D. J. Evaluation of the toxicity of ISIS 2302, a phosphorothioate oligonucleotide, in a 4-week study in CD-1 mice. *Antisense Nucleic Acid Drug Dev.* **7**, 473–481 (1997).
159. Heikenwalder, M. *et al.* Lymphoid follicle destruction and immunosuppression after repeated CpG oligodeoxynucleotide administration. *Nature Med.* **10**, 187–192 (2004).
160. Jason, T. L., Koropatnick, J. & Berg, R. W. Toxicology of antisense therapeutics. *Toxicol. Appl. Pharmacol.* **201**, 66–83 (2004).
161. Sparwasser, T. *et al.* Bacterial DNA causes septic shock. *Nature* **386**, 336–337 (1997).
162. Henry, S. P. *et al.* Complement activation is responsible for acute toxicities in rhesus monkeys treated with a phosphorothioate oligodeoxynucleotide. *Int. Immunopharmacol.* **2**, 1657–1666 (2002).
163. Galbraith, W. M., Hobson, W. C., Giclas, P. C., Schechter, P. J. & Agrawal, S. Complement activation and hemodynamic changes following intravenous administration of phosphorothioate oligonucleotides in the monkey. *Antisense Res. Dev.* **4**, 201–206 (1994).
164. Monteith, D. K. *et al.* Preclinical evaluation of the effects of a novel antisense compound targeting C-raf kinase in mice and monkeys. *Toxicol. Sci.* **46**, 365–375 (1998).

165. Henry, S. P., Novotny, W., Leeds, J., Auletta, C. & Kornbrust, D. J. Inhibition of coagulation by a phosphorothioate oligonucleotide. *Antisense Nucleic Acid Drug Dev.* **7**, 503–510 (1997).
166. Sheehan, J. P. & Lan, H. C. Phosphorothioate oligonucleotides inhibit the intrinsic tenase complex. *Blood* **92**, 1617–1625 (1998).
167. Krieg, A. M. CpG DNA: a pathogenic factor in systemic lupus erythematosus? *J. Clin. Immunol.* **15**, 284–292 (1995).
168. Hasegawa, K. & Hayashi, T. Synthetic CpG oligodeoxynucleotides accelerate the development of lupus nephritis during preactive phase in NZB x NZWF1 mice. *Lupus* **12**, 838–845 (2003).
169. Ichikawa, H. T., Williams, L. P. & Segal, B. M. Activation of APCs through CD40 or Toll-like receptor 9 overcomes tolerance and precipitates autoimmune disease. *J. Immunol.* **169**, 2781–2787 (2002).
170. Obermeier, F. *et al.* CpG motifs of bacterial DNA exacerbate colitis of dextran sulfate sodium-treated mice. *Eur. J. Immunol.* **32**, 2084–2092 (2002).
171. Ronaghy, A. *et al.* Immunostimulatory DNA sequences influence the course of adjuvant arthritis. *J. Immunol.* **168**, 51–56 (2002).
172. Katakura, K. *et al.* Toll-like receptor 9-induced type I IFN protects mice from experimental colitis. *J. Clin. Invest.* **115**, 695–702 (2005).
173. Boccaccio, G. L., Mor, F. & Steinman, L. Non-coding plasmid DNA induces IFN-γ *in vivo* and suppresses autoimmune encephalomyelitis. *Int. Immunol.* **11**, 289–296 (1999).
174. Quintana, F. J., Rotem, A., Carmi, P. & Cohen, I. R. Vaccination with empty plasmid DNA or CpG oligonucleotide inhibits diabetes in nonobese diabetic mice: modulation of spontaneous 60-kDa heat shock protein autoimmunity. *J. Immunol.* **165**, 6148–6155 (2000).
175. Wingender, G. *et al.* Systemic application of CpG-rich DNA suppresses adaptive T cell immunity via induction of IDO. *Eur. J. Immunol.* **36**, 12–20 (2006).
176. Mellor, A. L. *et al.* Cutting edge: CpG oligonucleotides induce splenic CD19+ dendritic cells to acquire potent indoleamine **2**, 3-dioxygenase-dependent T cell regulatory functions via IFN Type 1 signaling. *J. Immunol.* **175**, 5601–5605 (2005).
Together with reference 175, these studies reveal what seems to be an important counter-regulatory pathway that is induced by TLR9 agonists given systemically (intravenously) but not when the agonists are given via local routes. Further studies will be required to determine whether this effect is direct (as described by one of the investigators) or indirect (as reported by the other).
177. Chen, Y. *et al.* CpG DNA induces cyclooxygenase-2 expression and prostaglandin production. *Int. Immunol.* **13**, 1013–1020 (2001).
178. Ioannou, Y. & Isenberg, D. A. Current evidence for the induction of autoimmune rheumatic manifestations by cytokine therapy. *Arthritis Rheum.* **43**, 1431–1442 (2000).
179. Leadbetter, E. A. *et al.* Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* **416**, 603–607 (2002).
This seminal paper provided the first evidence that chromatin can activate TLR9, potentially triggering autoimmunity, as explored in further experiments from these and other investigators (references 180–183). Together, these studies point to potential therapeutic applications for TLR antagonists, which have been confirmed in references 184 and 185.
180. Viglianti, G. A. *et al.* Activation of autoreactive B cells by CpG dsDNA. *Immunity* **19**, 837–847 (2003).
181. Boule, M. W. *et al.* Toll-like receptor 9-dependent and-independent dendritic cell activation by chromatin-immunoglobulin G complexes. *J. Exp. Med.* **199**, 1631–1640 (2004).
182. Christensen, S. R. *et al.* Toll-like receptor 9 controls anti-DNA autoantibody production in murine lupus. *J. Exp. Med.* **202**, 321–331 (2005).
183. Means, T. K. *et al.* Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. *J. Clin. Invest.* **115**, 407–417 (2005).
184. Dong, L., Ito, S., Ishii, K. J. & Klinman, D. M. Suppressive oligodeoxynucleotides delay the onset of glomerulonephritis and prolong survival in lupus-prone NZB x NZW mice. *Arthritis Rheum.* **52**, 651–658 (2005).
185. Dong, L., Ito, S., Ishii, K. J. & Klinman, D. M. Suppressive oligonucleotides protect against collagen-induced arthritis in mice. *Arthritis Rheum.* **50**, 1686–1689 (2004).
186. Kawai, T. *et al.* Interferon-α induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. *Nature Immunol.* **5**, 1061–1068 (2004).
187. Uematsu, S. *et al.* Interleukin-1 receptor-associated kinase-1 plays an essential role for Toll-like receptor (TLR)7- and TLR9-mediated interferon-α induction. *J. Exp. Med.* **201**, 915–923 (2005).
188. Yeo, S. J., Yoon, J. G. & Yi, A. K. Myeloid differentiation factor 88-dependent post-transcriptional regulation of cyclooxygenase-2 expression by CpG DNA: tumor necrosis factor-α receptor-associated factor 6, a diverging point in the Toll-like receptor 9-signaling. *J. Biol. Chem.* **278**, 40590–40600 (2003).
189. Yeo, S. J., Gravis, D., Yoon, J. G. & Yi, A. K. Myeloid differentiation factor 88-dependent transcriptional regulation of cyclooxygenase-2 expression by CpG DNA: role of NF-κB and p38. *J. Biol. Chem.* **278**, 22563–22573 (2003).
190. Honda, K. *et al.* IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* **434**, 772–777 (2005).
191. Yang, K. *et al.* Human TLR-7-, -8-, and-9-mediated induction of IFN-α/β and-λ Is IRAK-4 dependent and redundant for protective immunity to viruses. *Immunity* **23**, 465–478 (2005).
192. Sun, C. M., Deriaud, E., Leclerc, C. & Lo-Man, R. Upon TLR9 signaling, CD5+ B cells control the IL-12-dependent Th1-priming capacity of neonatal DCs. *Immunity* **22**, 467–477 (2005).
193. Kerkmann, M. *et al.* Spontaneous formation of nucleic acid-based nanoparticles is responsible for high interferon-α induction by CpG-A in plasmacytoid dendritic cells. *J. Biol. Chem.* **280**, 8086–8093 (2005).
194. Marshall, J. D. *et al.* Novel chimeric immunomodulatory compounds containing short CpG oligodeoxyribonucleo tides have differential activities in human cells. *Nucleic Acids Res.* **31**, 5122–5133 (2003).
195. Fearon, K. *et al.* A minimal human immunostimulatory CpG motif that potently induces IFN-γ and IFN-α production. *Eur. J. Immunol.* **33**, 2114–2122 (2003).
196. Hartmann, G. *et al.* Rational design of new CpG oligonucleotides that combine B cell activation with high IFN-α induction in plasmacytoid dendritic cells. *Eur. J. Immunol.* **33**, 1633–1641 (2003).
197. Marshall, J. D. *et al.* Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. *J. Leukoc. Biol.* **73**, 781–792 (2003).
198. Kemp, T. J., Elzey, B. D. & Griffith, T. S. Plasmacytoid dendritic cell-derived IFN-α induces TNF-related apoptosis-inducing ligand/Apo-2L-mediated antitumor activity by human monocytes following CpG oligodeoxynucleotide stimulation. *J. Immunol.* **171**, 212–218 (2003).
199. Utaisincharoen, P. *et al.* CpG ODN enhances uptake of bacteria by mouse macrophages. *Clin. Exp. Immunol.* **132**, 70–75 (2003).
200. Klinman, D. M. Use of CpG oligodeoxynucleotides as immunoprotective agents. *Exp. Opin. Biol. Ther.* **4**, 937–946 (2004).
201. Efler, S. M., Zhang, L., Noll, B. O., Uhlmann, E. & Davis, H. L. Quantification of oligodeoxynucleotides in human plasma with a novel hybridization assay offers greatly enhanced sensitivity over capillary gel electrophoresis. *Oligonucleotides* **15**, 119–131 (2005).
202. Krieg, A. M. & Davis, H. L. *Vaccine Adjuvants: Immunological and Clinical Principles* (eds Hackett, C. J. & Harn, Jr. D. A.) 87–110 (Humana, Totowa, 2006).
203. Halperin, S. A. *et al.* Comparison of the safety and immunogenicity of hepatitis B virus surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide and a licensed hepatitis B vaccine in healthy young adults. *Vaccine* **24**, 20–26 (2006).
204. Lin, L., Gerth, A. J. & Peng, S. L. CpG DNA redirects class-switching towards 'Th1-like' Ig isotype production via TLR9 and MyD88. *Eur. J. Immunol.* **34**, 1483–1487 (2004).

Acknowledgements
I thank D. Arsemanlt for secretarial assistance.

Competing interests statement
The author declares competing financial interests: see Web version for details.

DATABASES
The following terms in this article are linked online to:
Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
CCR7 | IDO | IP10 | IRAK1 | IRF7 | MAGEA3 | MyD88 | p38 | SIGIRR | TLR9 | TRAF6 |

FURTHER INFORMATION
Oligonucleotide Therapeutics Society:
http://www.myots.org
Access to this links box is available online.

Manegold et al., Pfizer Poster 2005, Abstract 1131

Abstract 1131

Pfizer Oncology

Addition of PF-3512676 (CPG 7909) to a Taxane/Platinum Regimen for First-Line Treatment of Unresectable Non-Small Cell Lung Cancer (NSCLC) Improves Objective Response—Phase II Clinical Trial

C Manegold,[1] G Leichman,[2] D Gravenor,[3] D Woytowitz,[4] J Mezger,[5] G Albert,[6] T Schmalbach,[7] M Al-Adhami[7]

Abstract*

Background: A taxane/platinum regimen remains first-line treatment of stage IIIB/IV (unresectable) non-small cell lung cancer (NSCLC), yet the expected partial response is only 20% to 30%. Tumor response and survival have been significantly improved in many preclinical models with the addition of synthetic oligodeoxynucleotide PF-3512676 (CPG 7909), a Toll-like receptor 9 agonist with immunostimulatory activity. Previous trials have established the dosing ranges, biologic response, and safety of weekly subcutaneous PF-3512676.

Materials and Methods: To investigate the effect of adding PF-3512676 to standard chemotherapy for first-line treatment of stage IIIB/IV NSCLC, 112 chemotherapy-naive patients were randomized to receive four to six 3-week cycles of taxane and platinum chemotherapy alone (n = 37) or taxane and platinum plus 0.20 mg/kg subcutaneous PF-3512676 on weeks 2 and 3 of each cycle (n = 75). Baseline demographics were similar for the treatment arms; however, 85% of patients in the PF-3512676 arm had stage IV NSCLC versus 62% in the chemotherapy-alone arm. The phase II trial was conducted at 23 sites, and patients received study treatment until disease progression or unacceptable toxicity occurred. Primary endpoint was objective response rate (ORR), which was evaluated after cycles 2, 4, and 6 using Response Evaluation Criteria in Solid Tumors guidelines. Coded and blinded computed tomography scans from 91 of 112 patients underwent retrospective independent radiologic review. Ongoing secondary efficacy analyses included clinical benefit, time to tumor progression, duration of response, and survival; biomarker responses to PF-3512676 will be compared for responders and nonresponders in both arms.

Results: Data were available on all 112 patients for intention-to-treat response analysis. Investigator-evaluated ORR was 19% in the chemotherapy-alone arm and 37% in the PF-3512676 arm; independent radiologic review ORR was 11% versus 22%, respectively. Median overall survival was 6.8 months in the chemotherapy-alone and 12.8 months in the PF-3512676 arm, and Kaplan-Meier curves showed a trend toward improved overall survival in the PF-3512676 arm. One-year survival rates were 33% versus 50% in the chemotherapy-alone and PF-3512676 arms, respectively.

Conclusions: The data suggest that addition of weekly PF-3512676 to a taxane/platinum regimen for first-line treatment of NSCLC improves objective response. Confirmatory phase III trials are warranted to further document the clinical benefit of this new agent.

*Data presented in this poster are an important update of those in the published abstract.

Background

PF-3512676 (CPG 7909)

- Synthetic single-stranded oligodeoxynucleotide with unmethylated CpG motifs
- 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3'

PF-3512676 Mechanism of Action

- Rapid induction of innate immune response (Figure 1A)
  - Toll-like receptor 9 stimulation, which restores defective plasmacytoid dendritic cell function
  - Interferon alfa secretion (all types)
  - Natural killer (NK) and NKT-cell activation
- Later induction of adaptive immune response (Figure 1B)
  - Plasmacytoid dendritic cells induce type 1 helper T cells and promote NKT cells
  - B cells make antibodies

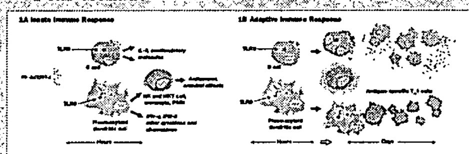


Figure 1.—Mechanism of action of PF-3512676, showing 1A) rapid induction of innate immune response and 1B) later induction of adaptive immune response. [illegible]

Proof-of-Concept Anticancer Activity

- More than 400 cancer patients have received at least 1 dose of PF-3512676
- Phase I/II trials of weekly subcutaneous PF-3512676 monotherapy, administered for a maximum of 24 weeks, have demonstrated evidence of antitumor activity in recurrent cutaneous T-cell lymphoma, metastatic malignant melanoma, and metastatic renal cell carcinoma (Table 1)

Table 1. Clinical Response to 24-Week Treatment With Subcutaneous PF-3512676 in Phase I/II Studies

Tumor type	Patients, n	Dose	CR + PR, n
Recurrent chronic cutaneous T-cell lymphoma*	37	0.08 to 0.36 mg/kg, 10 mg, and 25 mg	3 + 9
Metastatic malignant melanoma	105	6, 10, and 40 mg	0 + 3
Metastatic renal cell carcinoma*	35	0.08 to 0.81 mg/kg	0 + 2

CR = Complete response; PR = Partial response
*Ongoing.

Study Design

Treatment Scheme

- Randomized phase II trial in chemotherapy-naive patients with stage IIIB/IV non-small cell lung cancer (NSCLC) (Figure 2)
- 23 centers in the United States, Canada, and Germany (US—community oncology centers)

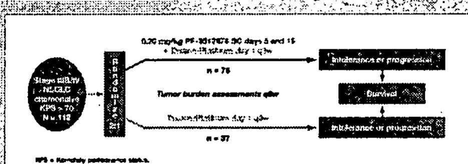


Figure 2.—Treatment scheme. NSCLC = Non-small cell lung cancer; SC = Subcutaneous; q3w = Every 3 weeks; q6w = Every 6 weeks.

- 3-week cycles: Week 1, all patients received a first-line taxane/platinum regimen; Weeks 2 and 3, patients assigned to receive PF-3512676 received 0.2 mg/kg PF-3512676 as a subcutaneous injection
- Study treatment continued until disease progression or unacceptable toxicity (maximum of 4 to 6 cycles)
- PF-3512676 was made available to patients randomized to the PF-3512676 combination arm who discontinued chemotherapy in the absence of disease progression (14 of 38 patients) at selected centers
- Patients randomized to chemotherapy alone were not permitted to receive PF-3512676 following disease progression

Treatment

Table 2. Treatment

	PF-3512676 + chemotherapy (n = 75)	Chemotherapy alone (n = 37)
Treatment regimen		
Paclitaxel 175 mg/m², cisplatin 75 mg/m²	[illegible]	[illegible]
Paclitaxel 175 mg/m², carboplatin AUC of 6	[illegible]	[illegible]
Docetaxel 75 mg/m², cisplatin 75 mg/m²	[illegible]	[illegible]
Docetaxel 75 mg/m², carboplatin AUC of 6	[illegible]	[illegible]
Number of chemotherapy treatment cycles		
Mean (min, max)	[illegible]	[illegible]
Chemotherapy dose reduction*		
Total	[illegible]	[illegible]
Reason		
CTC Grade 4 neutropenia	[illegible]	[illegible]
CTC Grade 3/4 thrombocytopenia	[illegible]	[illegible]
CTC Grade 3 non hematologic	[illegible]	[illegible]
CTC Grade 2 neuropathy	[illegible]	[illegible]
Other	[illegible]	[illegible]
PF-3512676 dosing		
Mean number of doses (min, max)	[illegible]	NA
PF-3512676 patients requiring dose reductions	[illegible]	NA

AUC = Area under the curve; min = Minimum; max = Maximum; CTC = Common toxicity criteria; NA = Not available.
*Protocol-required dose reduction for grade 4 hematologic events regardless of duration.

Results

Patient Demographics

- A total of 112 patients were treated
- Patient demographics were similar between treatment arms (Table 3)
  - More patients randomized to chemotherapy alone had stage IIIB NSCLC

Table 3. Summary of Patient Demographics

	PF-3512676 + chemotherapy (n = 75)	Chemotherapy alone (n = 37)
Age, years		
Mean (SD)	64 (10.5)	65.6 (8.8)
Median	66	67
Gender, n (%)		
Male	46 (61)	20 (54)
Female	29 (39)	17 (46)
Stage, n (%)		
IIIB	10 (13)	14 (38)
IV	64 (85)	23 (62)
KPS, n (%)		
≤ 70	10 (13)	5 (14)
> 70	65 (87)	32 (86)
Adenocarcinoma, n (%)		
Yes	35 (47)	19 (51)
No	40 (53)	18 (49)

SD = Standard deviation; KPS = Karnofsky performance status.

Response Rate (Primary Efficacy Endpoint)

- Physician evaluation: investigator-assessed confirmed and unconfirmed objective response rate by Response Evaluation Criteria in Solid Tumors guidelines (Figure 3)
- Independent radiology review: post hoc analysis of confirmed response; computed tomography scans collected retrospectively and evaluated for 91 patients (Figure 3)
- Early confirmation of objective response and routine imaging following discontinuation of chemotherapy treatment were not mandated by protocol

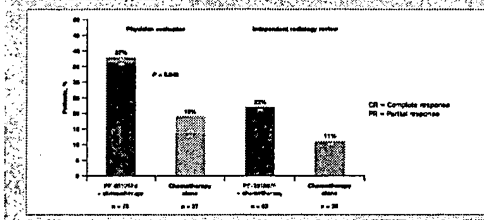


Figure 3.—Response rates, as assessed by physician evaluation or independent radiology review, for patients treated with PF-3512676 plus chemotherapy or chemotherapy alone.

Overall Survival

- Median survival was 12.8 months for patients receiving PF-3512676 in combination with chemotherapy versus 6.8 months for patients receiving chemotherapy alone (hazard ratio = 0.67; $P = .09$) (Figure 4)

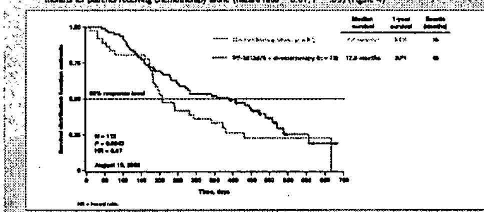


Figure 4.—Kaplan-Meier analysis of patients treated with PF-3512676 plus chemotherapy versus chemotherapy alone.

Safety

Adverse Events

- Local injection site reactions and systemic flu-like symptoms were the most common adverse events (Tables 4 and 5)
- 11 patients discontinued treatment because of adverse events possibly related to PF-3512676, including injection site reaction (5), systemic reaction (3), thrombocytopenia (1), pulmonary embolism (1), and gout (1)
- Despite a higher rate of grade 3 or 4 neutropenia in patients receiving PF-3512676 in combination with chemotherapy, the incidence of febrile neutropenia and treatment with growth factors was similar in the 2 arms. PF-3512676 is known to cause mild, reversible neutropenia that may reflect neutrophil margination
- Grade 3 or 4 thrombocytopenia events were numerically more common in patients receiving PF-3512676 in combination with chemotherapy but the only grade 4 event was in the chemotherapy-alone arm. No hemorrhagic events occurred in association with grade 3 or 4 thrombocytopenia
- 51% of patients receiving PF-3512676 in combination with chemotherapy received concomitant treatment for anemia compared with 41% of patients receiving chemotherapy alone

Table 4. Adverse Events (CTC Version 2.0)

Event term	All grades: PF-3512676 + chemotherapy	All grades: Chemotherapy alone	Grade 3: PF-3512676 + chemotherapy	Grade 3: Chemotherapy alone	Grade 4: PF-3512676 + chemotherapy	Grade 4: Chemotherapy alone
Fatigue	51	46	[illegible]	8	1	0
Weakness	13	16	3	[illegible]	1	0
Nausea	52	57	3	[illegible]	0	0
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Dehydration	15	19	3	14	[illegible]	0
Anemia	[illegible]	[illegible]	8	3	1	0
Neutropenia	[illegible]	[illegible]	19	19	49	22
Febrile neutropenia	[illegible]	[illegible]	3	0	0	3
Infections	[illegible]	[illegible]	1	5	0	0
Thrombocytopenia	[illegible]	[illegible]	15	0	0	3
Epistaxis	[illegible]	[illegible]	0	0	0	0
Hemoptysis	[illegible]	[illegible]	1	1	0	0

CTC = Common toxicity criteria.

Table 5. Reactions to PF-3512676

	Patients, %: All severities	Patients, %: Severe
Injection-site reactions		
Any	92	7
Induration	36	1
Pain	49	0
Erythema	85	3
Inflammation	37	1
Swelling	46	1
Systemic reactions		
Rigors	19	0
Pyrexia	37	0
Fatigue*	18	0
Arthralgia	7	0
Myalgia	9	0

Note: One patient on continuation therapy had an acute hypersensitivity/anaphylactic reaction.
*Transient fatigue considered to be associated with PF-3512676 administration.

Conclusions

- Investigator-assessed objective response rate (confirmed and unconfirmed) was 37% in patients receiving PF-3512676 in combination with chemotherapy versus 19% in patients receiving chemotherapy alone
- Independent radiology review demonstrated confirmed objective responses in 22% of patients receiving PF-3512676 in combination with chemotherapy and in 11% of patients receiving chemotherapy alone
- Median survival time was 12.8 months in patients receiving PF-3512676 in combination with chemotherapy versus 6.8 months in patients receiving chemotherapy alone
- 1-year survival rate was 50% in patients receiving PF-3512676 in combination with chemotherapy versus 33% in patients receiving chemotherapy alone
- PF-3512676 was well tolerated by most patients
- These data warrant confirmatory phase III trials

ACKNOWLEDGMENTS: C017 Study Group

Abstract*

Background: A taxane/platinum regimen remains first-line treatment of stage IIIB/IV (unresectable) non-small cell lung cancer (NSCLC), yet the expected partial response is only 20% to 30%. Tumor response and survival have been significantly improved in many preclinical models with the addition of synthetic oligodeoxynucleotide PF-3512676 (CPG 7909), a Toll-like receptor 9 agonist with immunostimulatory activity. Previous trials have established the dosing ranges, biologic response, and safety of weekly subcutaneous PF-3512676.

Materials and Methods: To investigate the effect of adding PF-3512676 to standard chemotherapy for first-line treatment of stage IIIB/IV NSCLC, 112 chemotherapy-naive patients were randomized to receive four to six 3-week cycles of taxane and platinum chemotherapy alone (n = 37) or taxane and platinum plus 0.20 mg/kg subcutaneous PF-3512676 on weeks 2 and 3 of each cycle (n = 75). Baseline demographics were similar for the treatment arms; however, 65% of patients in the PF-3512676 arm had stage IV NSCLC versus 62% in the chemotherapy-alone arm. The phase II trial was conducted at 23 sites, and patients received study treatment until disease progression or unacceptable toxicity occurred. Primary endpoint was objective response rate (ORR), which was evaluated after cycles 2, 4, and 6 using Response Evaluation Criteria in Solid Tumors guidelines. Coded and blinded computed tomography scans from 91 of 112 patients underwent retrospective independent radiologic review. Ongoing secondary efficacy analyses included clinical benefit, time to tumor progression, duration of response, and survival. Biomarker responses to PF-3512676 will be compared for responders and nonresponders in both arms.

Results: Data were available on all 112 patients for intention-to-treat response analysis. Investigator-evaluated ORR was 19% in the chemotherapy-alone arm and 37% in the PF-3512676 arm; independent radiologic review ORR was 11% versus 22%, respectively. Median overall survival was 6.8 months in the chemotherapy-alone and 12.8 months in the PF-3512676 arm, and Kaplan-Meier curves showed a trend toward improved overall survival in the PF-3512676 arm. One-year survival rates were 33% versus 50% in the chemotherapy-alone and PF-3512676 arms, respectively.

Conclusions: The data suggest that addition of weekly PF-3512676 to a taxane/platinum regimen for first-line treatment of NSCLC improves objective response. Confirmatory phase III trials are warranted to further document the clinical benefit of this new agent.

*Data presented in this poster are an important update of those in the published abstract.

Background

PF-3512676 (CPG 7909)

- Synthetic single-stranded oligodeoxynucleotide with unmethylated CpG motifs
- 5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′

PF-3512676 Mechanism of Action

- Rapid induction of innate immune response (Figure 1A)
  - Toll-like receptor 9 stimulation, which restores defective plasmacytoid dendritic cell function
  - Interferon alfa secretion (all types)
  - Natural killer (NK) and NKT-cell activation
- Later induction of adaptive immune response (Figure 1B)
  - Plasmacytoid dendritic cells induce type 1 helper T cells and promote NKT cells
  - B cells make antibodies

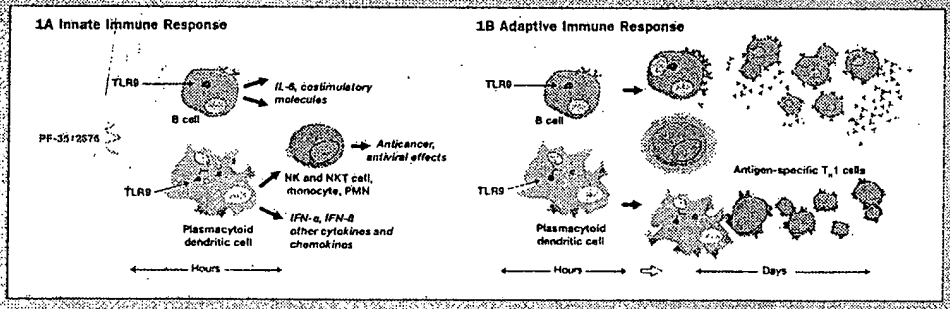


Figure 1.—Mechanism of action of PF-3512676, showing 1A) rapid induction of innate immune response and 1B) later induction of adaptive immune response. TLR-9 = Toll-like receptor 9; IL = Interleukin; NK = Natural killer; PMN = Polymorphonuclear neutrophil; IFN = Interferon; T_H1 = type 1 helper T cell.

Proof-of-Concept Anticancer Activity

- More than 400 cancer patients have received at least 1 dose of PF-3512676
- Phase I/II trials of weekly subcutaneous PF-3512676 monotherapy, administered for a maximum of 24 weeks, have demonstrated evidence of antitumor activity in recurrent cutaneous T-cell lymphoma, metastatic malignant melanoma, and metastatic renal cell carcinoma (Table 1).

Table 1. Clinical Response to 24-Week Treatment With Subcutaneous PF-3512676 in Phase I/II Studies

Tumor type	Patients, n	Dose	CR + PR, n
Recurrent chronic cutaneous T-cell lymphoma*	37	0.08 to 0.36 mg/kg, 10 mg, and 25 mg	3 + 9
Metastatic malignant melanoma	105	6, 10, and 40 mg	0 + 3
Metastatic renal cell carcinoma*	35	0.08 to 0.81 mg/kg	0 + 2

CR = Complete response; PR = Partial response.
*Ongoing.

Study Design

Treatment Scheme

- Randomized phase II trial in chemotherapy-naive patients with stage IIIB/IV non-small cell lung cancer (NSCLC) (Figure 2)
- 23 centers in the United States, Canada, and Germany (US –community oncology centers)

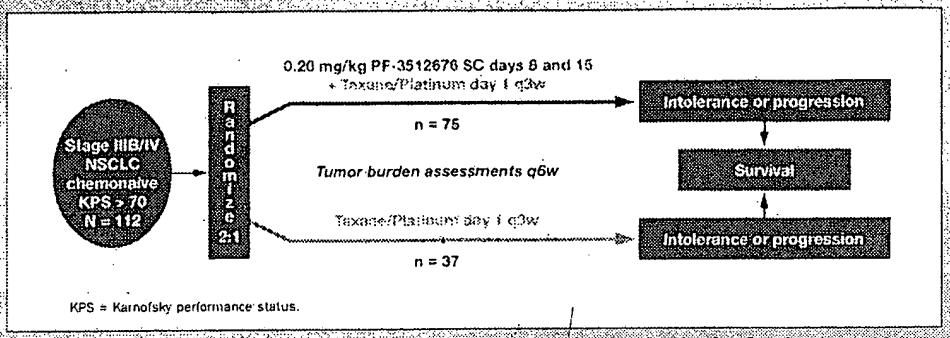


Figure 2.–Treatment scheme. NSCLC = Non-small cell lung cancer; SC = Subcutaneous; q3w = Every 3 weeks; q6w = Every 6 weeks

- 3-week cycles. Week 1, all patients received a first-line taxane/platinum regimen. Weeks 2 and 3, patients assigned to receive PF-3512676 received 0.2 mg/kg PF-3512676 as a subcutaneous injection
- Study treatment continued until disease progression or unacceptable toxicity (maximum of 4 to 6 cycles)
- PF-3512676 was made available to patients randomized to the PF-3512676 combination arm who discontinued chemotherapy in the absence of disease progression (14 of 38 patients) at selected centers
- Patients randomized to chemotherapy alone were not permitted to receive PF-3512676 following disease progression

Treatment

Table 2. Treatment

	PF-3512676 + chemotherapy (n = 75)	Chemotherapy alone (n = 37)
Treatment regimen		
Paclitaxel 175 mg/m²: cisplatin 75 mg/m²	4%	5%
Paclitaxel 175 mg/m²: carboplatin AUC of 6	36%	49%
Docetaxel 75 mg/m²: cisplatin 75 mg/m²	12%	16%
Docetaxel 75 mg/m²: carboplatin AUC of 6	48%	30%
Number of chemotherapy treatment cycles		
Mean (min, max)	4.2 (1, 8)	3.6 (1, 6)
Chemotherapy dose reduction*		
Total	33%	16%
Reason		
CTC Grade 4 neutropenia	18%	3%
CTC Grade 3/4 thrombocytopenia	6%	3%
CTC Grade 3 non hematologic	3%	5%
CTC Grade 2 neuropathy	2%	3%
Other	4%	3%
PF-3512676 dosing		
Mean number of doses (min, max)	11 (1, 45+)	NA
PF-3512676 patients requiring dose reductions	5% (All due to injection site reactions)	NA

AUC = Area under the curve; min = Minimum; max = Maximum; CTC = Common toxicity criteria; NA = Not available.
*Protocol-required dose reduction for grade 4 hematologic events regardless of duration.

Results

Patient Demographics

- A total of 112 patients were treated
- Patient demographics were similar between treatment arms (Table 3)
  - More patients randomized to chemotherapy alone had stage IIIB NSCLC

Table 3. Summary of Patient Demographics

	PF-3512676 + chemotherapy (n = 75)	Chemotherapy alone (n = 37)
Age, years		
Mean (SD)	64 (10.5)	65.6 (8.8)
Median	65	67
Gender, n (%)		
Male	46 (61)	20 (54)
Female	29 (39)	17 (46)
Stage, n (%)		
IIIB	10 (13)	14 (38)
IV	64 (85)	23 (62)
KPS, n (%)		
≤ 70	10 (13)	5 (14)
> 70	65 (87)	32 (86)
Adenocarcinoma, n (%)		
Yes	35 (47)	19 (51)
No	40 (53)	18 (49)

SD = Standard deviation; KPS = Karnofsky performance status.

Response Rate (Primary Efficacy Endpoint)

- Physician evaluation: investigator-assessed confirmed and unconfirmed objective response rate by Response Evaluation Criteria in Solid Tumors guidelines (Figure 3)
- Independent radiology review: post hoc analysis of confirmed response; computed tomography scans collected retrospectively and evaluated for 91 patients (Figure 3)
- Early confirmation of objective response and routine imaging following discontinuation of chemotherapy treatment were not mandated by protocol

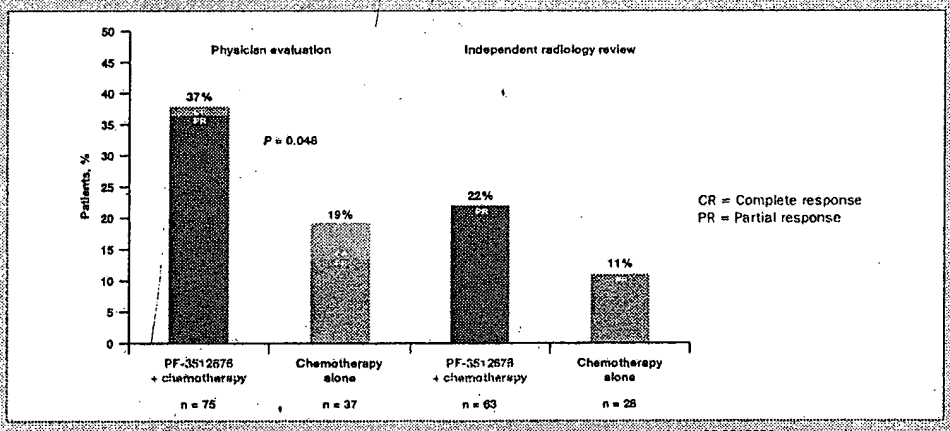
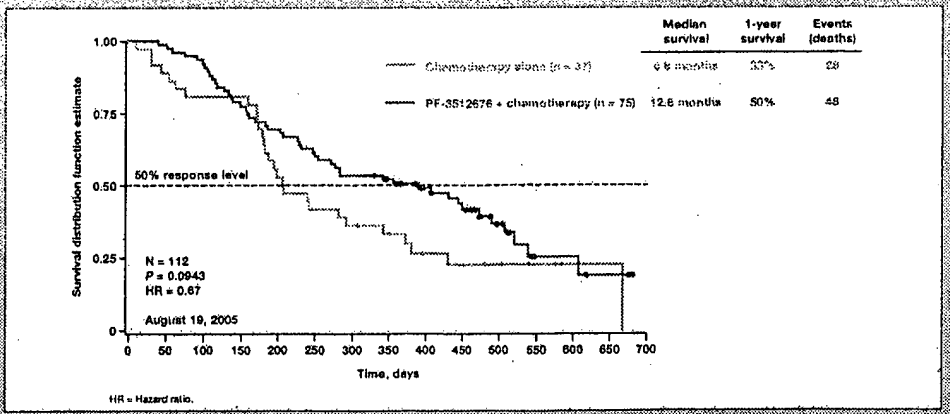


Figure 3.–Response rates, as assessed by physician evaluation or independent radiology review, for patients treated with PF-3512676 plus chemotherapy or chemotherapy alone.

Overall Survival

- Median survival was 12.8 months for patients receiving PF-3512676 in combination with chemotherapy versus 6.8 months for patients receiving chemotherapy alone (hazard ratio = 0.67; $P = .09$) (Figure 4)



HR = Hazard ratio.

Figure 4.–Kaplan-Meier analysis of patients treated with PF-3512676 plus chemotherapy versus chemotherapy alone.

Safety

Adverse Events

- Local injection site reactions and systemic flu-like symptoms were the most common adverse events (Tables 4 and 5)
- 11 patients discontinued treatment because of adverse events possibly related to PF-3512676, including injection site reaction (5), systemic reaction (3), thrombocytopenia (1), pulmonary embolism (1), and gout (1)
- Despite a higher rate of grade 3 or 4 neutropenia in patients receiving PF-3512676 in combination with chemotherapy, the incidence of febrile neutropenia and treatment with growth factors was similar in the 2 arms. PF-3512676 is known to cause mild, reversible neutropenia that may reflect neutrophil margination
- Grade 3 or 4 thrombocytopenia events were numerically more common in patients receiving PF-3512676 in combination with chemotherapy, but the only grade 4 event was in the chemotherapy-alone arm. No hemorrhagic events occurred in association with grade 3 or 4 thrombocytopenia
- 51% of patients receiving PF-3512676 in combination with chemotherapy received concomitant treatment for anemia compared with 41% of patients receiving chemotherapy alone

Table 4. Adverse Events (CTC Version 2.0)

	Patients,%					
	All grades		Grade 3		Grade 4	
Event term	PF-3512676 + chemotherapy	Chemotherapy alone	PF-3512676 + chemotherapy	Chemotherapy alone	PF-3512676 + chemotherapy	Chemotherapy alone
Fatigue	51	46	5	8	1	0
Weakness	13	16	3	8	1	0
Nausea	62	57	3	5	0	0
Diarrhea	39	35	7	8	0	0
Vomiting	25	35	1	5	0	0
Abdominal pain	7	8	1	0	1	0
Peripheral neuropathy	16	14	3	0	0	0
Anorexia	27	30	0	5	0	0
Dehydration	15	19	3	14	1	0
Anemia			8	3	1	0
Neutropenia			19	19	49	22
Febrile neutropenia			3	0	0	3
Infections			1	5	0	0
Thrombocytopenia			15	0	0	3
Epistaxis			0	0	0	0
Hemoptysis			1	1	0	0

CTC = Common toxicity criteria.

Table 5. Reactions to PF-3512676

	Patients, %	
	All severities	Severe
Injection-site reactions		
Any	92	7
Induration	36	1
Pain	49	0
Erythema	85	3
Inflammation	37	1
Swelling	48	1
Systematic reactions		
Rigors	19	0
Pyrexia	37	0
Fatigue*	19	0
Arthralgia	7	0
Myalgia	5	0

Note: One patient on continuation therapy had an acute hypersensitivity/anaphylactic reaction.
*Transient fatigue considered to be associated with PF-3512676 administration.

Conclusions

- Investigator-assessed objective response rate (confirmed and unconfirmed) was 37% in patients receiving PF-3512676 in combination with chemotherapy versus 19% in patients receiving chemotherapy alone
- Independent radiology review demonstrated confirmed objective responses in 22% of patients receiving PF-3512676 in combination with chemotherapy and in 11% of patients receiving chemotherapy alone
- Median survival time was 12.8 months in patients receiving PF-3512676 in combination with chemotherapy versus 6.8 months in patients receiving chemotherapy alone
- 1 year survival rate was 50% in patients receiving PF-3512676 in combination with chemotherapy versus 33% in patients receiving chemotherapy alone
- PF-3512676 was well tolerated by most patients
- These data warrant confirmatory phase III trials

ACKNOWLEDGMENTS: C017 Study Group